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N-dihydrogalactochitosan as immune and direct antitumor agent amplifying the effects of photodynamic therapy and photodynamic therapy-generated vaccines

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ABSTRACT

It is becoming apparent that to obtain robust and prolonged antitumor responses in cancer immunotherapy, appropriate adjunct agents promoting both tumor antigen delivery and immune rejection enhancement are critically required. The semisynthetic biopolymer N-dihydrogalactochitosan (GC) is emerging as a promising such candidate. In the present study, the effects of GC were investigated when combined with cancer vaccines generated by photodynamic therapy (PDT) using mouse tumor model SCCVII (squamous cell carcinoma). The adjunct GC treatment was found to enhance therapeutic benefit obtained with PDT vaccine, while reducing the numbers of myeloid-derived suppressor cells. Another important property of GC is promoting directly the death of SCCVII cells sustaining injury from PDT mediated by various photosensitizers. This effect is extended to cells treated by cryoablation therapy (CAT) performed by exposure to −80 °C. A capacity of GC for preferential binding to PDT treated cells was demonstrated using fluorescence microscopy. *In vitro* testing with specific caspase-1 inhibitor revealed a pro-survival role of this enzyme in membrane lipid repair mechanisms following combined PDT plus GC treatment. In conclusion, GC represents a uniquely promising adjunct for various PDT protocols, photothermal and similar rapid tumor-ablating therapies.

1. Introduction

Immune modulators, capable of breaking immunotolerance in tumor microenvironment, promoting robust and long-lasting antitumor adaptive immune responses while acting as efficient therapeutic delivery tools and direct antitumor agents, have become recognized as crucial component of cancer therapy [1]. One candidate with potential of being clinically established as such immune simulant is N-dihydrogalactochitosan (GC), a semisynthetic cationic carbohydrate polymer and derivative of chitin (an abundant natural polysaccharide) [2,3]. A number of pre-clinical and clinical studies have authenticated the validity of GC (previously known also as glycated chitosan) as an effective adjunct to tumor photothermal therapy (PTT) and other local ablation therapies [2,4–7]. This agent was found to be ingested by macrophages and dendritic cells stimulating their immune activities [8,9]. It was suggested that GC can enhance the uptake and presentation of tumor antigens or play the role of a damage-associated molecular pattern (DAMPs) [8]. The advantageous properties of GC include water solubility, sterile filterability, biocompatibility, low toxicity and capability to serve as a physiologically compatible carrier [3,8].

In an earlier report [2], GC was shown to augment the effectiveness of tumor photodynamic therapy (PDT), a clinically established modality for treatment of tumors employing photosensitizing drugs for the production of cytotoxic oxygen species upon absorbing light focused on targeted lesions [10]. Tumor-localized thermal and oxidative stress, produced by PTT and PDT, respectively, trigger a common threat of proteostasis impairment due to proteotoxic injury that evokes in tumor cells a homeostatic evolutionary well preserved canonic protection mechanisms based on stress signaling networks [11]. These signal transduction pathways include immunogenic cell death signaling encompassing the expression and trafficking of DAMPs and abundant

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exposure of tumor-associated antigens (TAA) including highly immunogenic normally cryptic antigens. This characteristic vigorous immune signaling activity enables both PTT and PDT to generate a potent immune responses against treated cancers [5,12,13].

Novel findings we recently reported on GC when used in combination with PTT include the influence on immunoregulatory cell levels in treated tumors, preferential binding of GC to PTT-damaged tumor cells compared to untreated cells, and direct enhancement of PTT-mediated tumor cell killing [14]. Given the above described similarities between PTT and PDT, the present study was performed to gain insight into the aspects of GC activity responsible for its efficacy as the potentiator of tumor control by PDT.

2. Materials and methods

2.1. Cell culture and tumors

For *in vitro* experiments, SCCVII cells originally obtained from murine squamous cell carcinoma [15] were cultured in alpha minimal essential medium supplemented with 10% fetal bovine serum (Gibco by Life Technologies, Burlington ON, Canada). These cells were used for preparing PDT-generated cancer vaccine and inoculating SCCVII tumors implanted subcutaneously into lower dorsal site of 7–9 week old syngeneic C3H/HeN mice. These tumors serve as a recognized model of head and neck cancer of spontaneous origin lacking cogent immunogenicity [16]. The procedure protocols with mice were approved by the Animal Care Committee of the University of British Columbia.

2.2. PDT light treatment

In vitro PDT with photosensitizers chlorin e6 (Frontier Scientific, Logan UT), Photofrin (Axcan Pharma, Mont-Saint-Hilaire QC, Canada), or Temoporfin (Biolitec Research GmbH, Jena, Germany) were performed as described previously. For ce6-PDT, SCCVII cells were incubated with 1 μg/ml ce6 concentration for 30 min in serum-free medium followed by 665 ± 10 nm light treatment of 1 J/cm^2 (30 mW/cm^2) [17]. Treatment of cells with Photofrin-PDT was performed by incubating the cells with Photofrin 20 μg/ml for 18 h and then exposing to 630 ± 10 nm light (1 J/cm^2; 40 mW/cm^2), while Temoporfin-PDT was done by cell incubation with Temoporfin (0.1 μg/ml) for 18 h with subsequent illumination (650 ± 10 nm, 1 J/cm^2 with 38 mW/cm^2) [18]. The light was produced by a high throughput fiber illuminator model FB-QTH-3 equipped with 150 W QTH lamp (Sciencetech Inc., London ON, Canada) employing interchangeable interference filters, and with liquid light guide (model 77638, Oriel Instruments, Stratford CT) used for light delivery.

2.3. Cryoablation treatment

In the experimental *in vitro* protocol modeling tumor cryoablation therapy (CAT) [19], predetermined numbers of SCCVII cells (usually 1–2 × 10^5) were pelleted by centrifugation in 14 ml polystyrene round-bottom tubes, the supernatants carefully removed, and the tubes placed in a −80 °C incubator for pre-set time intervals. Immediately thereafter, the cells were re-suspended in a warm growth medium and aliquots used for cell colony plating. The extent of tumor cell killing by CAT was determined by colony formation assay.

2.4. PDT vaccine

The tumors were treated 5 days after inoculation when they reached around 5 mm in largest diameter. The vaccine preparation procedure was described in detail earlier [17]. Briefly, after the treatment by ce6-PDT as described above, SCCVII cells were returned to culture conditions and kept in a specially enriched animal-component free chemically defined serum- and protein free medium (#14650C, Sigma Chemical Co., St. Louis MO) for 16 h at 37 °C. The cells were then exposed to lethal dose of x-rays (60 Gy) and immediately thereafter injected peritumorally (2 × 10^7 cells/mouse in 0.15 ml) into SCCVII tumor-bearing mice. In PDT vaccine plus GC protocol, 0.1 ml of the original 1% GC preparation was included in 0.15 ml volume administering the vaccine, and (where indicated) the same volume injected peritumorally 2, 4, and 6 days later. In control GC alone group, mice were injected peritumorally with 0.1 ml of 1% GC. The agent GC (N-dihydrogalactochitosan) was provided by Immunophotonics Inc. (St. Louis MO). Vaccine response was monitored by measuring changes in tumor size using a caliper. Cyclophosphamide (50 mg/kg) was injected into mice i.p. 4 days after vaccination as a part of standard vaccine protocol because of its capacity to block immunoregulatory T cells [20].

2.5. MDSCs analysis

As explained previously [21], the levels of myeloid-derived suppressor cell (MDSC) populations in mice were determined by obtaining cell suspensions from spleens and staining the splenocytes for flow cytometry. The cells of interest were identified by antibodies phycoerythrin-cyanine5-conjugated rat anti-mouse GR1 (eBioscience, San Diego CA) and phycoerythrin-conjugated anti-mouse CD11b produced in mouse (Santa Cruz Biotechnology Inc., Dallas TX). Flow cytometry was performed using a Coulter Epics Elite ESP (Coulter Electronics, Hialeah FL) and total numbers of MDSCs per spleen were derived from the yields of GR1$^+$CD11b$^+$ cells from known weighs of tissue.

2.6. Cell survival

Standard clonogenic assay was used for determining the extent of cell killing by tested treatments. Immediately after PDT *in vitro*, known numbers of treated cells were plated in 60 mm Petri dishes and incubated 7 days for the formation of macroscopic survivor colonies. They were then stained in aqueous malachite green solution (5 mg/ml) and counted. Also used in this *in vitro* study were the specific caspase-1 inhibitor INF·4E, and sterol regulatory element binding protein (SREBP) inhibitor fatostatin A (both from Tocris, Bio-Techne Canada, Oakville ON, Canada), as well as purified annexin V (BioLegend, San Diego CA). The inhibitors INF·4E and fatostatin A were added immediately after colony plating (both 20 μM) and left during the initial 4 h of clonal growth. Cells were exposed to annexin V (sodium azide removed from its original solution using a 10 kDa molecular centrifugal filter) contained in Annexin V Binding Buffer (PharMingen Canada, Mississauga ON, Canada). For GC treatment, this agent was diluted directly from its original 1% aqueous solution into the cell medium with plated colonies.

Viability loss of cancer cells in SCCVII tumors treated by PDT vaccine was assessed by digesting the excised tumors to obtain single cell suspensions for plating for colony formation. Plating efficiency and cell yield were used for expressing the findings as relative colony forming units; the latter corresponds to the equivalent values obtained with untreated tumors [22].

2.7. Fluorescence microscopy

For preparing fluorescence-tagged GC, FITC dissolved in dehydrated methanol was mixed with GC and then FITC-GC purified by filtration through 100 kDa filters, as reported earlier [8]. The stock solution used for the experiments was 0.5% GC containing 0.5 mM bound FITC (1:25 molecular ratio). Immediately after PDT treatment, cells were placed over a glass slide in Petri dish and incubated 20 min with purified annexin V (5 μg per 1000 cells) or control annexin V binding buffer) at room temperature. The cells were then left in growth medium with GC-FITC (250 μg GC/ml plus 25 μM FITC) or with 25 μM FITC (Toronto Research Chemicals, Toronto ON, Canada) without GC in a 37 °C incubator for 3 h. The attached cells were then fixed, slides coverslipped, and fluorescent cell images at 20× magnification acquired by a

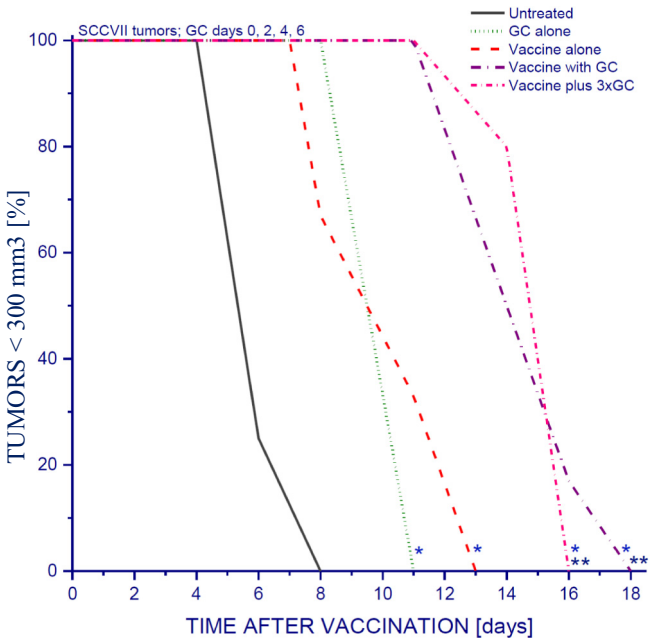

Fig. 1. Response of mouse SCCVII tumors to PDT vaccine combined with GC. Mice bearing SCCVII tumors were treated with vaccine prepared from PDT-treated SCCVII cells. Twenty millions of these cells were used for vaccination by a peritumoral injection. For single GC treatment, 0.1 ml of its 1% GC preparation was included in the vaccine injection volume or injected peritumorally with GC alone group. For multiple treatments, additional 0.1 ml injections of 1% GC were administered peritumorally on days 2, 4, and 6 after initial vaccination. As a component of the standard vaccine protocol, the mice were administered cyclophosphamide (50 mg/kg i.p.) at 4 days post vaccine injection. Responses are presented as percentages of mice remaining with tumors smaller than 300 mm^3 at different time-points after vaccination. *Statistically significant difference in response ($p < 0.05$) compared to untreated control group; **statistically significant difference in response ($p < 0.05$) compared to vaccine alone group.

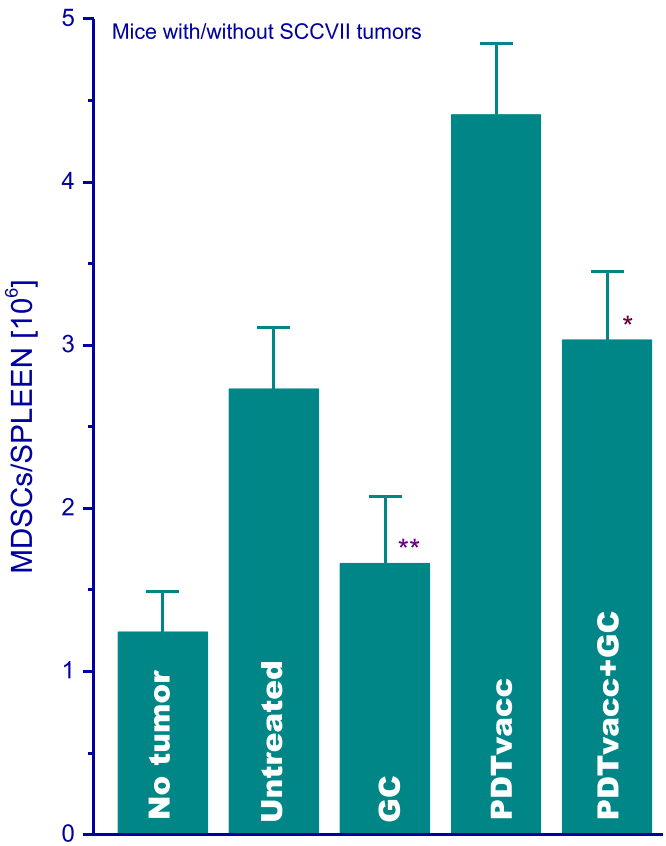

Fig. 2. The impact of PDT vaccine and GC on spleen MDSC levels in treated mice. Mice bearing SCCVII tumors were treated with PDT vaccine with or without GC as described for Fig. 1. Their splenocytes, collected from the mice sacrificed 72 h later, were stained for flow cytometry and analyzed for determining the levels of MDSCs. The results are presented as total number of MDSCs per spleen. $N = 4$, bars are SD. *Statistically significant difference ($p < 0.05$) compared to the value for PDT vaccine only group; **statistically significant difference ($p < 0.05$) compared to the value for untreated tumor control.

fluorescence microscope Axio Imager Z1 (Carl Zeiss Canada Ltd., Toronto ON, Canada) equipped with FITC filter set. The images were captured using a low light ProxOnyx USB camera (Teledyne e2v Ltd., Milpitas CA) with no gains (analog = 1, digital = 1) and 12 milliseconds exposure time. For the final data, expressed as arbitrary FITC fluorescence intensity units per cell, the background fluorescence value obtained from cells not exposed to GC-FITC was subtracted.

2.8. Statistical analysis

The data analysis was performed for obtaining the means and standard deviations. Statistical evaluation for designating p values was based on Mann-Whitney test. The adapted confidence interval for identifying differences between individual experimental groups was 95%, with the threshold for statistical significance set at 5%.

3. Results

Benefit of combining PDT vaccine treatment with GC for controlling growth of SCCVII tumors is shown in Fig. 1. The cut-off value of 300 mm^3 for tumor volume in the ordinate axis was chosen because below this size the tumors tend to be sensitive to therapeutic intervention while larger lesions have progressed too much to still be affected. As shown in a number of our previous studies [20,21,23,24], the vaccine prepared from PDT-treated SCCVII cells produced a significant retardation of the growth rate of SCCVII tumors. Interestingly, a single peritumoral GC injection without vaccine treatment also produced a detectable temporary delay in SCCVII tumor growth in this experiment. A pronounced additional delay in tumor progression was observed by incorporating GC into PDT vaccine delivery. No significant additional therapeutic impact was gained by extending the GC application by injections at 2, 4, and 6 days post vaccine treatment (Fig. 1).

Spleens were obtained from mice sacrificed 72 h after treatments including PDT vaccine, GC alone, and PDT vaccine plus single GC treatment. The levels of MDSCs among splenocytes were determined using antibody staining and flow cytometry analysis. The results show that both PDT vaccine and GC had a significant influence on the number of these immunoregulatory cells present in spleens (Fig. 2). As shown earlier [21], untreated mice bearing SCCVII tumors had a characteristic rise in this population compared to tumor-free mice. Remarkably, this was largely eliminated by a single GC alone treatment. As also reported earlier [21], PDT vaccine treatment provoked a noticeable increase in the size of MDSC population in the spleen (as a regulatory element coupled with the induction of immune response) but this effect was blocked when PDT vaccine was combined with GC.

To obtain additional insights on the response of SCCVII tumors to PDT vaccine with or without GC, tumors were excised from sacrificed mice at either 3 or 5 days post initial treatment and the extent of parenchymal cancer cells killing assessed by colony formation assay. The results reveal that the PDT vaccine treatment alone produced death of about two thirds of cancer cells at the 3-day post-treatment interval followed by one more log of cell killing within the next 2 days (Fig. 3). This effect was further boosted by combining PDT vaccine with GC, as it

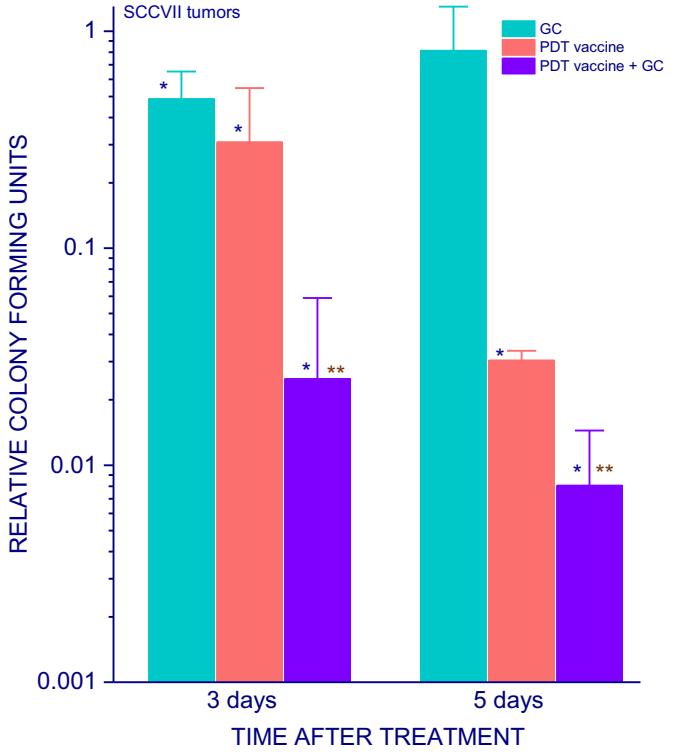

Fig. 3. Survival of cancer cells in SCCVII tumors at 3 and 5 days after treatment with PDT vaccine, GC, or their combination. Mice bearing SCCVII tumors were treated with PDT vaccine with or without GC as described for Fig. 1, and tumors excised from sacrificed mice 3 or 5 days after treatment. Cancer cell survival was determined relative to the findings with untreated tumors based on plating efficiency and yield of cells obtained from the tumors. N = 4, bars are SD. *Statistically significant difference in survival ($p < 0.05$) compared to untreated tumor group; **statistically significant difference in survival ($p < 0.05$) compared to PDT vaccine only group.

produced an additional log of cell killing at the 3-day time-point and further half a log two days later. Interestingly, treatment with GC alone also affected significantly tumor cell survival, as around half a log of killing was documented 3 days later. However, this effect of GC was lost at 5 days post-treatment presumably reflecting the regrowth of these fast growing tumors.

As immunomodulating agent, GC should exhibit its antitumor effects only under *in vivo* conditions, and in the absence of immune cells *in vitro* no impact on tumor cell survival would be expected. However, GC proved effective in augmenting the death rate of SCCVII cells treated with PDT *in vitro*, and this was demonstrated with 3 different photosensitizers (Fig. 4). In these experiments, cells were plated for clonogenic assay immediately after PDT treatment with or without GC present throughout the colony growth. The only exception was a group of samples with GC present only during the initial 4 h after colony plating, which served to demonstrate that the full effect of GC was already expressed during this initial interval (Fig. 4a). Both tested doses of GC, 100 μg/ml (GC1) and 500 μg/ml (GC2) were effective in amplifying cell killing rate of temoporfin-PDT (Fig. 4a), as well as Photofrin-PDT and ce6-PDT (Fig. 4b). However, the higher GC dose was consistently more effective. In additional testing, it was demonstrated that GC has a similar impact on cells treated by CAT (Fig. 4c). Comparable effects were found with cells treated by low dose CAT (80% survival range, CAT dose 1) and high dose CAT (< 1% survival, CAT dose 2). With two GC concentrations used in this case, GC high (835 μg/ml) was more effective than GC salient (167 μg/ml). Manifestly, exposure to GC had no significant effect on tumor cell survival without PDT/CAT treatment (Fig. 4b/c).

Since exposure to GC selectively affected PDT-treated but not PDT-untreated cells it seems pertinent to examine possible preference of GC binding to these two cell populations. For this purpose, SCCVII cells were immediately after treatment by Temoporfin-PDT exposed to FITC conjugated GC (250 μg/ml) in growth medium and left to attach to glass slides. The same was done with control untreated SCCVII cells. The slides were collected 3 h later and the cells were after fixation analyzed by florescence microscopy. The obtained cell images showed fluorescence patterns consistent with the localization of GC-FITC on cellular surface (Fig. 5a). The overall results clearly demonstrate that there was much greater binding of GC-FITC to PDT-treated than to untreated cells (Fig. 5b). The results in this figure also reveal that this binding was indeed mediated by GC (since it cannot be attributed to free FITC) and that it can be blocked by prior exposure to annexin V.

To investigate whether the observed direct enhancement of PDT-mediated cell kill by GC involves caspase-1 activity, the electrophilic warhead-based designed small molecule INF·4E that selectively inhibits this cell death enzyme was included in samples with PDT-treated cells exposed to GC during the initial 4 h after plating their colonies. The results show that the survival of cells treated by PDT alone or by PDT plus GC was significantly decreased in the presence of 20 μM INF·4E that was non-toxic to untreated cells (Fig. 6a). This reveals that caspase-1 supports survival of cells trying to cope with damage from PDT or PDT plus GC treatment. To support this finding, further testing was done with fatostatin A, since it specifically inhibits SREBPs transcription factors regulating membrane lipid biosynthesis that are responsible for pro-survival caspase-1 activity [25]. The results show that SREBP inhibition has a comparable effect to caspase-1 inhibition reducing the survival of cells treated by PDT with or without GC (Fig. 6b). Viability of cells untreated by PDT was not affected by the same fatostatin A treatment (not shown). The indicated prospect of an additive effect by using both INF·4E and fatostatin A, although not corroborated statistically, could reflect the outcome of a more complete blockage of cell membrane biogenesis.

The possibility that the direct cell kill-promoting effect of GC is prompted by its binding to negatively charged membrane phospholipids becoming exposed on PDT-treated cells was confirmed by pre-exposing these cells to annexin V before the treatment with GC. This pre-treatment with annexin V completely blocked the effect of GC on the survival of cells treated by either moderately or more severely lethal PDT dose (0.5 and 1.0 J/cm^2, respectively) (Fig. 7).

4. Discussion

To obtain a robust and long-lasting antitumor efficacy in cancer immunotherapy, the availability of an appropriate adjuvant achieving both superlative antigen delivery and enhancement of the raised immune response is critically important. The present study establishes that GC acts as a highly effective non-toxic adjunct to locally rapidly tumor-ablating cancer therapies such as PTT, PDT, and CAT. The first revelation that GC can interact effectively with standard PDT treatment of tumors *in situ* was reported in an earlier study [2]. It was shown that PDT treatment of mouse mammary sarcomas or lung adenocarcinomas combined with peritumoral GC injection (which by itself produced no tumor cures) resulted in an enhancement of curative outcome compared to PDT alone. Since GC is known to act as immunostimulant, we wanted to determine whether such benefit with GC is also found with a PDT application (vaccine) that relies predominantly on eliciting antitumor immune response. Indeed, the present work demonstrates that the therapeutic benefit is broadened to include the application of GC as adjunct in therapeutic PDT vaccine protocols. The acquired capacity of immune rejection of treated tumors by these vaccines results after they secure the recognition of tumor antigens and is known to be carried out by cytotoxic T cells [13,17]. The optimal impact was attained with a single GC treatment immediately after PDT (either incorporated into the vaccine volume or as a separate peritumoral injection) and no

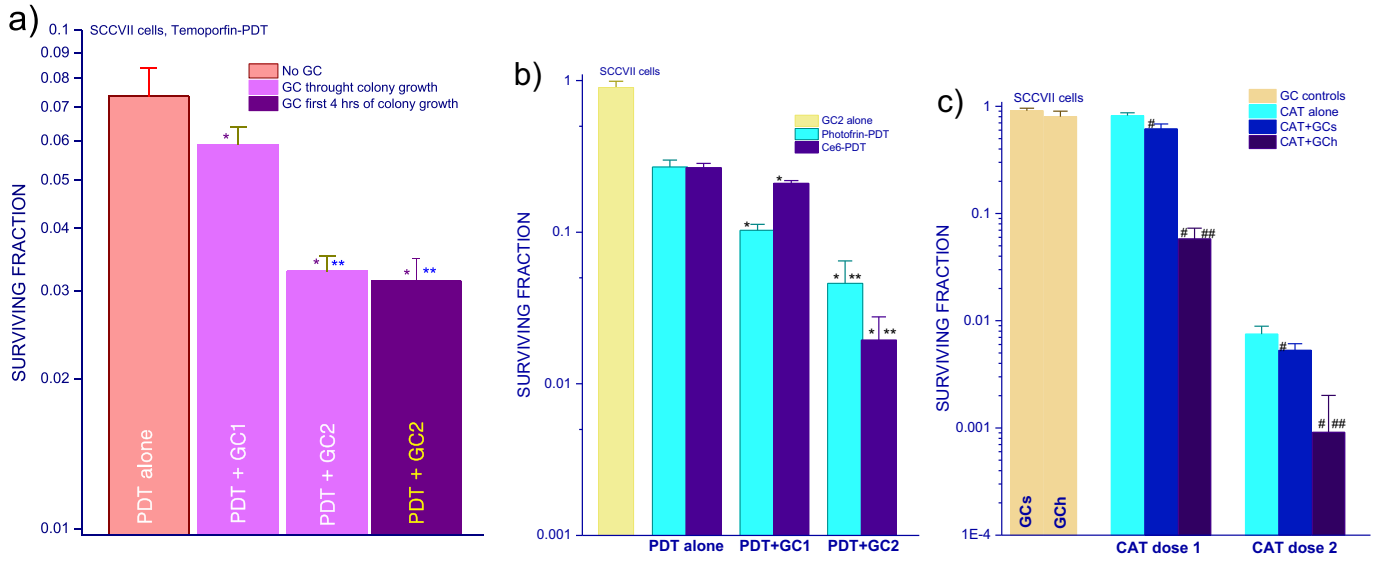

Fig. 4. GC decreases survival of PDT- or CAT-treated SCCVII cells *in vitro*. *In vitro* cultured SCCVII cells were treated either by PDT (a,b), or CAT (c) and then plated immediately for colony survival. For temoporfin-PDT, cells were exposed to 0.2 μg/ml temoporfin concentration for 24 h and then to 1 J/cm^2 of 650 ± 10 nm light. In other protocols, cells were exposed to Photofrin (20 μg/ml) and 1 h later to 1 J/cm^2 of 630 ± 10 nm light, or to chlorin e6 (1.5 μg/ml) followed 30 min later by 665 ± 10 nm light. For CAT, cell pellets were exposed to -80 °C for 2 min (CAT dose 1) or 4 min (CAT dose 2). In most cases, GC1 (100 μg/ml) or GC2 (500 μg/ml) were added at the time of colony plating and left throughout clonal growth; the exception was the group of samples with GC left with cells for only the initial 4 h after colony plating. With CAT, GC doses were 167 μg/ml (GC salient, GCs) or 835 μg/ml (GC high, GCh). The results show cell survival (triplicate samples means). *Statistically significant difference in survival ($p < 0.05$) compared to PDT alone group; **statistically significant difference in survival ($p < 0.05$) compared to PDT + GC1 group; #statistically significant difference in survival ($p < 0.05$) compared to CAT alone group; ##statistically significant difference in survival ($p < 0.05$) compared to CAT + GCs group.

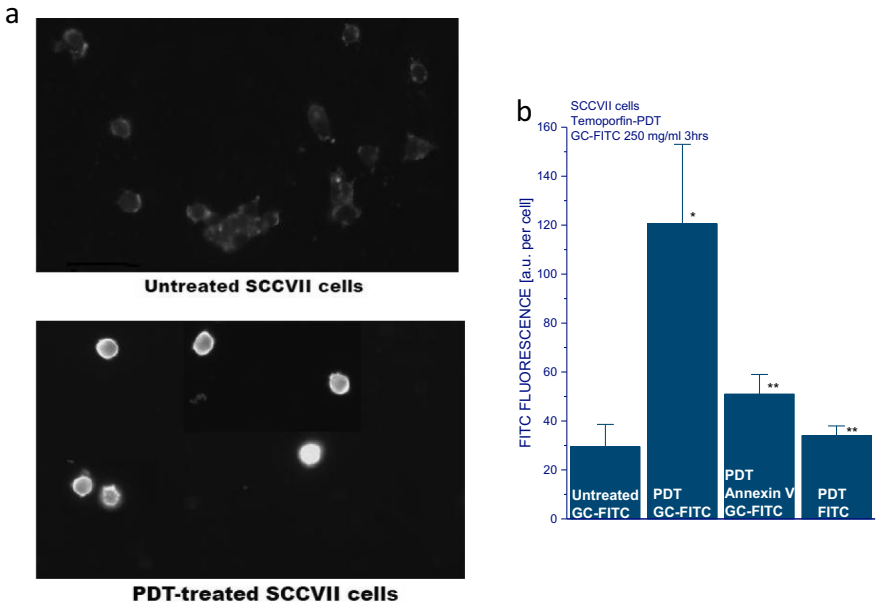

Fig. 5. Binding of GC to PDT-treated cells. Immediately after PDT exposure described for Fig. 4a, SCCVII cells resuspended in growth medium containing GC-FITC (250 μg/ml) were left 3 h in a 37 °C-incubator to attach to glass slides. Before the GC-FITC exposure, the cells were incubated 20 min with annexinV (5 μg per 1000 cells) or control buffer. A control group of PDT-treated cells were exposed to the equivalent concentration of FITC without GC. The slides were then collected and prepared for fluorescence microscopy. (a) Examples of fluorescent images of cells incubated with GC-FITC. (b) Levels of cell-bound GC based on GC-associated FITC fluorescence determined from fluorescent cell images. The data are presented as means of GC-FITC fluorescence intensity in arbitrary units per cell ($N = 20$). *Statistically significant difference compared to the value for untreated cells ($p < 0.05$); **statistically significant difference compared to the value for PDT plus GC-FITC (no Annexin V) group.

further improvement was registered with additional multiple injections (Fig. 1). The therapeutic impact of GC as adjunct to PDT observed as tumor growth retardation was, at least during the initial therapy time interval, closely corresponding to decreasing survival of parenchymal cancer cells (Fig. 3). The latter is bound to reflect not only direct killing of these cells by PDT and GC but also their elimination by elicited antitumor immune activity. Interestingly, the data in the present study reveal that GC has the capacity of enhancing this immune response not only by directly promoting activation of DCs and macrophages [8,9] but also indirectly by affecting the involvement of MDSCs.

The unique property of GC is the capacity for antitumor intervention by both indirect means through the modulation of host immune cell activity and a direct interaction with targeted cancer cells [14], Fig. 4. Moreover, there is an intimate cross-talk between these two types of action with the lethal effect manifesting as immunogenic cell death (ICD) [11]. Thermal and oxidative stress, induced by PTT/CAT and PDT, respectively, was shown to elicit immune responses against tumors treated by these therapies and this seems to be associated with the induction of ICD [11,26–28].

Advancements in CAT protocols (*e.g.* high-definition ultrasound guided introduction of miniaturized cryoprobes) have spurred a renewed interest in this modality for treatment of not only various skin cancers but also tumors within the liver, lungs, bone, kidney, prostate and breasts [19]. The results of the present report suggest that cancer

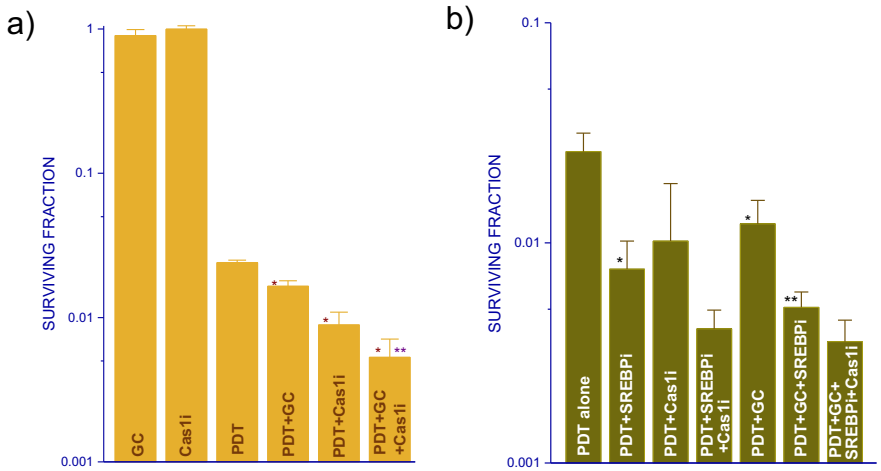

Fig. 6. The role of caspase-1in GC-mediated enhancement of cell viability loss following PDT treatment. Cultured SCCVII cells were treated by PDT (temoporfin 0.25 μg/ml for 24 h plus 1 J/cm^2 of 650 ± 10 nm light) followed by exposure to GC (0.25 mg/ml) and/or caspase-1 inhibitor INF·4E (20 μM) for initial 4 h upon colony plating (a). Additionally, the effect of SREBP inhibitor fatostatin A (20 μM), present also for the initial 4 h upon plating, was evaluated (b). The results show cell survival (triplicate samples means) determined by a standard colony formation assay. *Statistically significant difference compared to the value for PDT alone group ($p < 0.05$); **statistically significant difference compared to the value for PDT plus GC group.

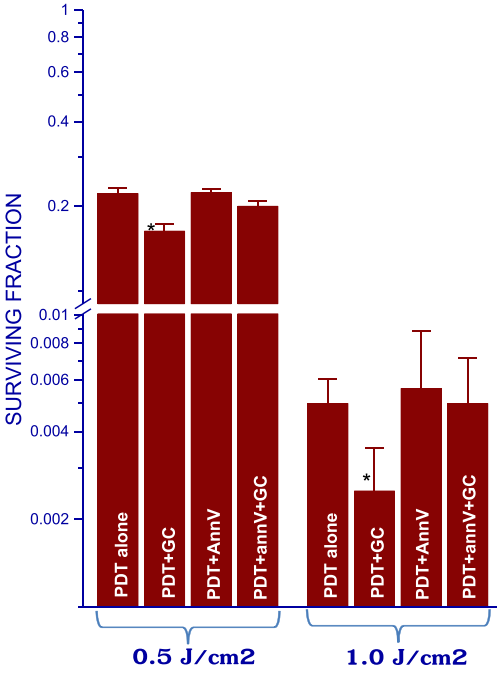

Fig. 7. The pre-exposure to annexin V blocks GC-mediated enhancement of cell viability loss following PDT treatment. Cultured SCCVII cells were treated by PDT (temoporfin 0.25 μg/ml for 24 h plus 0.5 or1 J/cm^2 of 650 ± 10 nm light). Immediately after PDT light treatment, the cells were exposed to annexin V (5 μg per 1000 cells) or control buffer for 20 min at room temperature followed promptly by addition of growth medium with or without GC (0.25 mg/ml) that was kept on throughout the colony growth. The results show cell survival (triplicate samples means) determined by a standard colony formation assay. *Statistically significant difference compared to the value for PDT alone group ($p < 0.05$).

cell injuries inflicted by CAT are in the same category as those caused by PTT or PDT when it comes to the interaction with GC responsible for clinical benefit achieved with this agent. Further investigations into common features of cellular thermal stress caused by elevated temperatures (PTT) and cold thermal energy (CAT) are in progress in our laboratory.

The previously reported direct cancer killing effect of GC against PTT-treated cells was demonstrated in this work to be closely emulated in the interaction of GC with cells treated by PDT mediated by various photosensitizers as well as tumor cells sustaining CAT-mediated injury (Fig. 4). For this effect, GC presence was critical during the initial hours following PDT and a direct positive correlation was found with GC dose.

Our earlier finding that GC has a disposition of preferentially binding to PTT-treated cells compared to their untreated counterparts [14] was extended in this study with the evidence of association with cells affected by PDT treatment. Tumor-ablating insult delivered by these therapies leaves cancer cells affected with a wide range of cytoplasmic and membrane damage [10] including surface exposure of negatively charged membrane phospholipids like phosphatidylserine [23]. We have suggested that positively-charged GC could have a high affinity for binding to such structures [14]. This is now substantiated by the demonstration that pre-exposure to annexin V obstructs GC binding to PDT-treated cells (Fig. 3) and blocks GC mediated enhancement of PDT-based cell killing by (Fig. 7). Due to its high predisposition for binding to acidic phospholipids with especially strong affinity for phosphatidylserine [29], annexin V was evidently competing with GC for the same interaction sites on the cell surface.

Importantly, potent pro-immune agents like calreticulin and heat shock protein 70 also show a high avidity for preferential binding to PDT-treated tumor cells [30,31]. The ability of these agents and GC to bind to tumor cells sustaining injury/stress could be instrumental for their delivery to cancerous lesions for performing effective antitumor activity and their prolonged retention at the targeted site. Such binding could trigger a preferential engulfment by phagocytes to secure effective disposal of these cellular targets as well as tumor antigen processing and presentation for the adaptive immune response. Furthermore, with GC there is an additional course of action, namely, the direct killing effect resulting presumably from the interference with repair of damaged membrane structures [14]. In the clinic, this could translate into enhanced killing at the margin of ablation and improved local clearance with reduced chance of tumor recurrence.

Similarly as with PTT plus GC combination [14], cellular survival for PDT + GC was not significantly altered in presence of neither FAS agonist Kp7-6 nor caspase-8 inhibitor *Z*-IETD-fmk (data not shown). This suggests that, similarly as with PTT, GC binding to PDT-treated cells does not engage death receptors and extrinsic apoptosis pathway signaling. A further correlation with the effect of GC on PTT is the implication of a pro-survival involvement of caspase-1 revealed by the reduced survival of PDT plus GC treated cells in presence of the inhibitor of this enzyme INF·4E (Fig. 6). Such role of caspase-1 could be achieved by its modulation of lipid metabolism pathways known to be mediated by the induction of SREBPs (central regulators of cellular membrane biogenesis) [25], which would facilitate the repair of damaged cellular membranes whose integrity was additionally disrupted by GC binding. This scenario is substantiated by the finding that specific inhibition of SREBPs (even limited to the initial 4 h following PDT treatment) has effects similar to INF·4E and drastically reduces the survival of cells treated by PDT with or without combined GC (Fig. 6b).

In conclusion, GC is established as a uniquely promising adjunct not

only for various PDT protocols but also emerges as a highly favorable choice for joint use with various tumor-ablating therapies because of sharing similar underlying antitumor mechanisms in such combinations. Such therapies comprise thermal- (including PTT, CAT, radiofrequency ablation, microwave ablation, and high intensity focused ultrasound) or electric force-based modalities [32].

Declaration of Competing Interest

Tomas Hode and Samuel S.K. Lam declare a conflict of interest as employees of Immunophotonics Inc. which manufactures N-dihydrogalactochitosan. The other authors declare no conflict of interest.

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