



Inhibition of human breast cancer growth by GCPTM (genistein combined polysaccharide) in xenogeneic athymic mice: involvement of genistein biotransformation by β -glucuronidase from tumor tissues

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Abstract

The role of β -glucuronidase in genistein biotransformation was investigated in a human breast cancer MDA-MB-231 xenogeneic athymic mouse model. Genistein combined polysaccharide (GCPTM), a genistein aglycone rich functional food supplement was used in these experiments. Tumor-bearing mice were subjected to oral administration of GCPTM for 28 days. GCPTM treatment significantly inhibited tumor growth. Induction of apoptosis by GCPTM treatment was related to activation of cleavage of poly(ADP-ribose)polymerase, induction of the p21 protein expression and reduction of cyclin B1 expression in the tumor tissues. Genistein exists as a glucuronide conjugate in normal organ tissues, and the conjugated genistein lacks the physiological activity of the aglycone. Tumor tissues contain large amounts of β -glucuronidase, the enzyme that converts the genistein β -glucuronide conjugate into genistein aglycone. The resulting genistein aglycone exerts its chemopreventive activities, including the induction of apoptosis in tumor tissues, and, finally, leads to tumor growth inhibition.

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1. Introduction

Epidemiologic as well as laboratory studies have revealed that soy isoflavones exert chemopreventive effects on several types of human cancer [1,2]. Isoflavones identified in soybeans are mainly glycosides, including genistin, daidzin and glycitin, that are

conjugated with glucose [3]. Their active forms are deglycosylated aglycones, such as genistein, daidzein and glycitein [4]. Genistein is a well-known molecule which exerts multiple biological activities. These include inhibition of tyrosine kinases [5], inactivation of DNA topoisomerase II [6], anti-angiogenesis [7] and cell growth arrest by interfering with signal transduction cascades [8]. Soy isoflavone glycosides are degraded into their aglycones mainly through metabolism by gut microflora [9,10]. Soy isoflavone aglycones are absorbed faster and in larger amounts

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than their glucosides [11]. Genistein is absorbed in the gut, taken up by the liver and excreted in the bile as its 7-*O*- β -glucuronide. The principal metabolites of genistein in the circulation are glucuronide and sulfate conjugates. Conjugated genistein is excreted into urine or bile. Genistein exerts its biological activities when the conjugated genistein is degraded into an aglycone by β -glucuronidase [12–14].

Endo- β -*D*-glucuronidase (commonly referred to as heparanase) is an extracellular matrix enzyme that degrades heparan sulfate and heparan sulfate proteoglycans, the major components of extracellular matrix and vascular basement membrane [15,16]. Therefore, the endo- β -*D*-glucuronidase is thought to accelerate cancer invasion and metastasis. This enzyme is expressed in a low level in normal tissues, but is expressed to a higher extent during inflammation, angiogenesis or malignant transformation [17]. These results may be due to differential rates of genistein metabolism in normal and abnormal tissues.

In order to clarify the relationship between genistein metabolism and β -glucuronidase in tumor and normal tissues, we have used a fermented extract of soybean isoflavone and basidiomycetes which contains a high amount of physiologically active isoflavone aglycones. Genistein is the major component, and the mixture named genistein combined polyssacharide (GCPTM). In the present study, we have investigated the effects of GCPTM on tumor proliferation in athymic nude mice bearing human breast cancer cells (MDA-MB-231). We have further investigated the metabolism of genistein and its relationship with the β -glucuronidase activity in tumor and in normal tissues.

2. Materials and methods

2.1. Materials

GCPTM is a functional supplement developed by the Amino Up Chemical Co. Ltd. (Sapporo, Japan) in September 2000. The detailed information of GCPTM is provided in the website: <http://www.aminoup.co.jp>. One gram of GCPTM contains 116 ± 8.4 mg of genistein, 28.5 ± 5.4 mg of daidzein, 13.5 ± 2.6 mg of glycitein and about 3% of insoluble polysaccharides from basidiomycetes [18].

2.2. Methods

2.2.1. Animals

BALB/cA Jcl-nu athymic mice (female, 6 weeks of age) were purchased from CLEA Japan Co. Ltd. (Tokyo, Japan). Animal care and treatment conformed to the standards relating to the care and management, etc. of experimental animals by Japanese Association for Experimental Animal Science (JALAS).

2.2.2. Cell culture

The MDA-MB-231 human breast cancer cell line was kindly provided by Dr. Surh of Seoul National University. The cells were maintained with Dulbecco's minimum essential medium containing 10% fetal bovine serum.

2.2.3. Xenogeneic transplantable tumor models

MDA-MB-231 human breast cancer cells (5×10^6) were inoculated subcutaneously into 6-week-old BALB/cA Jcl-nu female athymic mice. Control mice ($n = 5$) were fed aseptically prepared CE-2 diet. CE-2 is a standard CLEA rodent diet purchased from CLEA Japan Co. Ltd. (Tokyo, Japan). The detailed composition information is described in the website: <http://www.clea-japan.co.jp/siryoc1-3.htm>. Experimental mice received CE-2 diet supplemented with 1% GCPTM ($n = 5$). The GCPTM was given during the entire period of the experiment. The body weights and food consumption were measured on every third day. The growth of tumors was measured after they first became palpable by the formula of length \times width \times depth \times 0.5236 (mm³). All the mice were sacrificed on the 28th day after the tumor inoculation. The tumors were removed and the tumor weights were measured.

2.2.4. High-performance liquid chromatograph (HPLC) analysis for genistein content in tumor and normal tissues

HPLC analysis for genistein measurement was carried out as described by Franke et al. [19] and Song et al. [20] with some modifications. Briefly, tumor and liver and colon tissue samples were homogenized and adjusted to equal content of protein (Micro BCA protein assay reagent kit, Pierce, Rockford, IL) with 0.1 M acetate buffer (pH 5.0). A total of 300 μ l of tissue samples were mixed with an equal volume of

acetate buffer. The samples were treated with 5 μ l β -glucuronidase (Sigma, St. Louis, MO) at 37 °C for 1 h or were untreated and next extracted with 600 μ l of ethyl acetate. After centrifugation at 3000 rpm for 10 min, 500 μ l of the upper layer were removed and dried by gentle stream of N₂ gas. HPLC analyses were carried out using an Hitachi Chromatography Instrument. (Advanced HPLC System Manager Model 7000 with an auto-sampler Model L-7200 and a UV-Vis detector Model L-7420, Hitachi Co. Ltd., Tokyo, Japan). The concentration of genistein was calculated the genistein standard curve (Sigma, St. Louis, MO). The concentrations of other aglycon isoflavone including daidzein and glycitein were measured as well.

2.2.5. Measurement of β -glucuronidase activity in tumor and normal tissues

β -Glucuronidase activity was measured according to the previously reported method with some modifications [21]. The tumor tissues or non-tumor organs such as liver, lungs, kidneys, and brain were homogenized and their protein content was normalized to 100 μ g/ml (Micro BCA protein assay reagent kit, Pierce, Rockford, IL) with 0.1 M acetate buffer (pH 5.0). A standard curve was prepared with 1 unit of β -glucuronidase for degrading 100 μ g/ml of phenolphthalein mono- β -glucuronic acid as a substrate. A total of 100 μ l samples (10 μ g protein) were added to the 96-well plate and incubated at 37 °C for 1 h. The reaction was stopped by adding 100 μ l of 0.2 M Na₂CO₃. The absorbance of each well was measured by use of a microplate reader (Hitachi, Tokyo, Japan) at 492 nm.

2.2.6. Western blot analysis

Tumor tissue lysates were suspended with 100 μ l lysis buffer (150 mM NaCl, 0.5% Triton-X100, 50 mM Tris-HCl, pH 7.4, 1 μ g/ml aprotinin and 100 μ g/ml phenylmethylsulfonyl fluoride). The samples were prepared with 5 \times SDS loading buffer. The equal amounts of proteins were loaded onto the gel. The SDS-PAGE was run at 200 V, 60 mA for 2 h. The gel was transferred to a PDVF membrane (Bio-Rad Laboratories, CA) at 200 mA for 2 h, and the membrane was blocked with 5% nonfat-dry milk-PBST for 2 h. The rabbit polyclonal antibodies used are as follows: poly(ADP-ribose)polymerase (PARP): sc-7150; p21^{WAF1}, and cyclin B1. The second antibody used

in this analysis was anti-rabbit IgG-horseradish peroxidase. The primary antibodies were diluted 1000 times. The membrane was incubated with the primary antibody in 10 ml dilution buffer with gentle agitation overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for an additional 1 h at room temperature. The membrane was then incubated with 10 ml ECL detection reagent (Amersham Pharmacia Biotec Inc., Piscataway, NJ), and exposed to X-ray film.

2.3. Statistical analysis

All statistical tests were performed using the unpaired *t*-test. Significant differences for tumor growth curve, tumor weights, food intake, body weight gain, β -glucuronidase activities and genistein concentration in tumor and normal tissues were assessed between control and GCPTM-treated group. *P* < 0.05 was considered to be a significant difference. Values in the text and figures are represented as means \pm S.D.

3. Results

3.1. GCPTM inhibited human breast cancer growth in vivo

Diet consumption, genistein intake, body weight gain, tumor growth curve and tumor weight were measured. The average diet consumption was 3.5 \pm 0.5 g per mouse per day. The mouse body weight increased from 21.94 \pm 0.26 to 30.42 \pm 2.17 g for the control group (*n* = 5) and from 22.84 \pm 0.77 to 27.72 \pm 1.39 g for the GCPTM-treated group (*n* = 5) during the period of the study. There was no significant difference between the two groups. The average genistein consumption was 4.06 \pm 0.27 mg per mouse per day (162.4 \pm 10.8 mg/kg body weight per day). The tumors grew palpably about 14 days after tumor inoculation. The tumors in the control group supplied with CE-2 diet grew quickly after 21 days and enlarged several times within 2 weeks. The tumors in the GCPTM-treated group grew more slowly after 21 days and their growth almost stopped in the next week. The tumor growth curves were significantly different between the control and GCPTM-treated

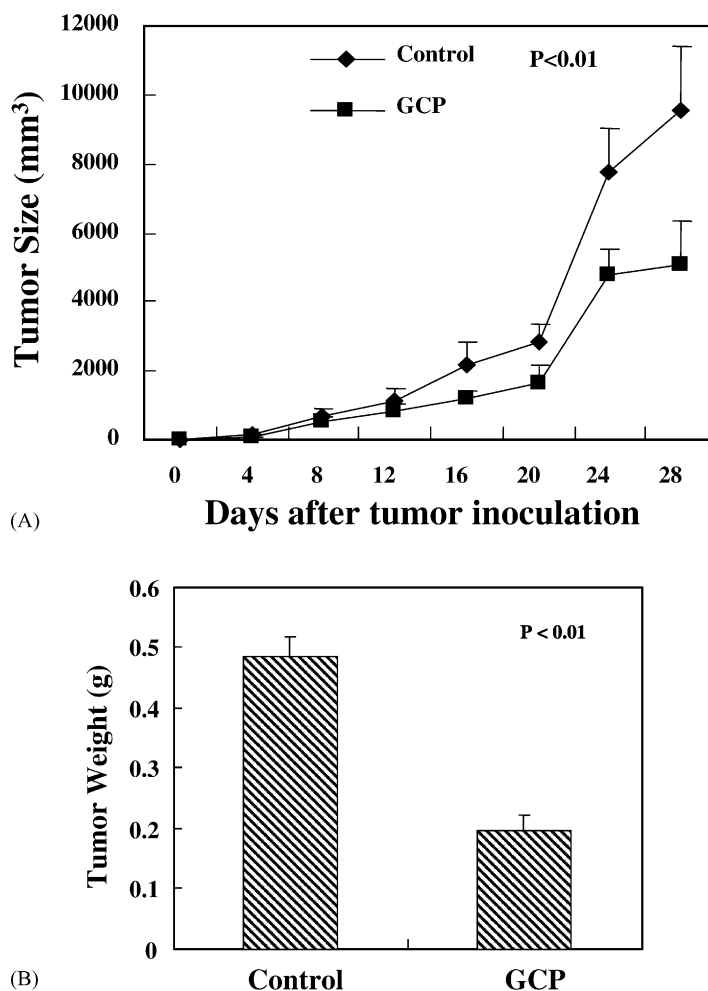


Fig. 1. Effects of GCPTM treatment on proliferation of (A) and weight of (B) human breast cancer cells inoculated into nude mice. MAD-MB-231 human breast cancer cells (5×10^6) were inoculated subcutaneously into BALB/cA Jcl-nu athymic mice. The mice were fed aseptically prepared CE-2 diet (control) or CE-2 diet supplemented with 1% GCPTM. The GCPTM was provided throughout the experiment. The growth and weight of tumors were measured every third day after they were initially found to be palpable. All the mice were sacrificed on the 28th day after tumor inoculation. The data are presented as mean \pm S.D. ($n = 5$).

groups starting at day 21 ($P < 0.01$, Fig. 1A). The tumor weights obtained on day 28 after tumor inoculation showed significant differences between the control and GCPTM-treated groups (the average tumor weights in control and GCPTM-treated group were 0.484 ± 0.035 g and 0.196 ± 0.026 g, respectively, ($P < 0.01$, Fig. 1B). These data indicate that GCPTM might be a potential anti-carcinogenic food supplement for both tumor prevention and treatment.

3.2. Effects of GCPTM on cleavage of PARP, induction of p21^{WAF1}, and reduction of cyclin B1 expression in human breast tumor tissues of xenogeneic athymic mice

Apoptosis-related proteins, including PARP, p21^{WAF1}, and cyclin B1, were detected in the tumor tissues of human breast cancer treated with/without GCPTM. The results showed that GCPTM treatment caused cleavage of PARP, induced expression of

p21^{WAF1} protein and reduced cyclin B1 expression in human breast tumor tissues of xenogeneic athymic mice (Fig. 2). These data suggest that apoptosis was induced in the GCPTM treated tumor tissues.

3.3. The levels of aglycone genistein were higher in tumor tissues than those of any other organs

Free genistein that existed in tissues was detectable by HPLC without pre-treatment with β -glucuronidase, while the conjugated genistein was only detectable after pretreatment with β -glucuronidase. In this study, genistein concentration in various tissues was determined after pretreatment with β -glucuronidase or without pretreatment for the respective aglycone and conjugated forms. Neither aglycone genistein nor conjugated genistein was detectable in the tumor or other organ tissues of control group (data not shown). The amount of aglycone genistein in GCPTM-treated tumor tissues was significantly higher than those in normal liver and colon tissues (tumor: $78.42 \pm 23.17 \mu\text{g}/\text{mg}$ protein; liver: $21.49 \pm 1.91 \mu\text{g}/\text{mg}$ protein; colon:

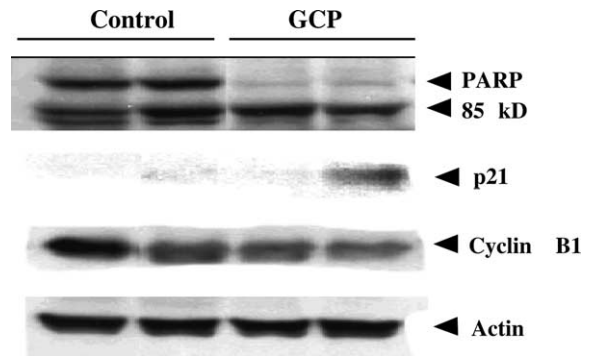


Fig. 2. Effects of GCPTM administration on expression of PARP, p21^{WAF1} and cyclin B1 proteins in human breast tumor tissues. Tumors were removed from control and GCPTM treated tumor-bearing mice on the day 28 after tumor inoculation. The tumor tissues were homogenized by an ultrasonic processor and lysed. The SDS-PAGE was run as described in Section 2. Actin was used as a loading control.

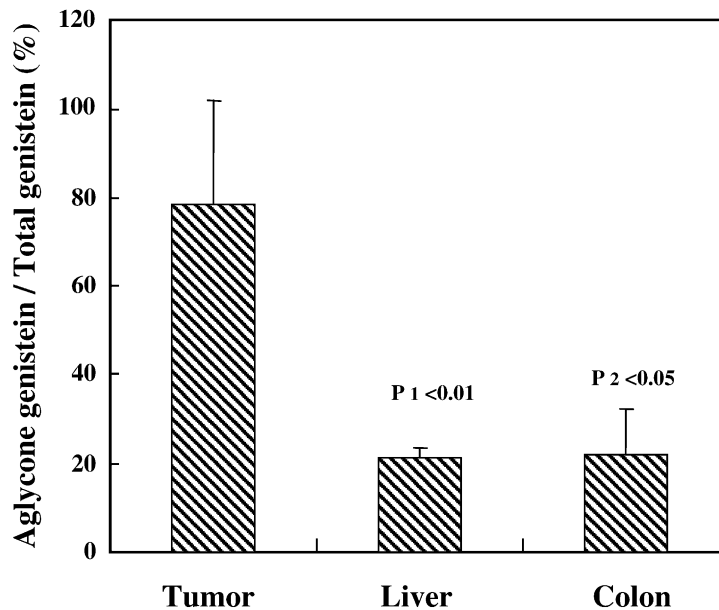


Fig. 3. Comparison of levels of aglycone genistein and conjugated genistein contained in tumor tissues and in other organs. Tumor or liver and colon tissues from GCPTM-treated tumor-bearing mice were homogenized and adjusted to an equal content of protein. The samples were treated with/without $5 \mu\text{l}$ of β -glucuronidase and extracted with $600 \mu\text{l}$ ethyl acetate. HPLC analysis for genistein measurement was conducted as described in Section 2. The genistein concentrations were expressed as $\mu\text{g}/\text{mg}$ protein. The percentages of aglycone genistein in total genistein (aglycone and conjugated) are expressed. The data are presented as mean \pm S.D. ($n = 5$). $P_1 < 0.01$ for tumor vs. liver and $P_2 < 0.05$ for tumor vs. colon.

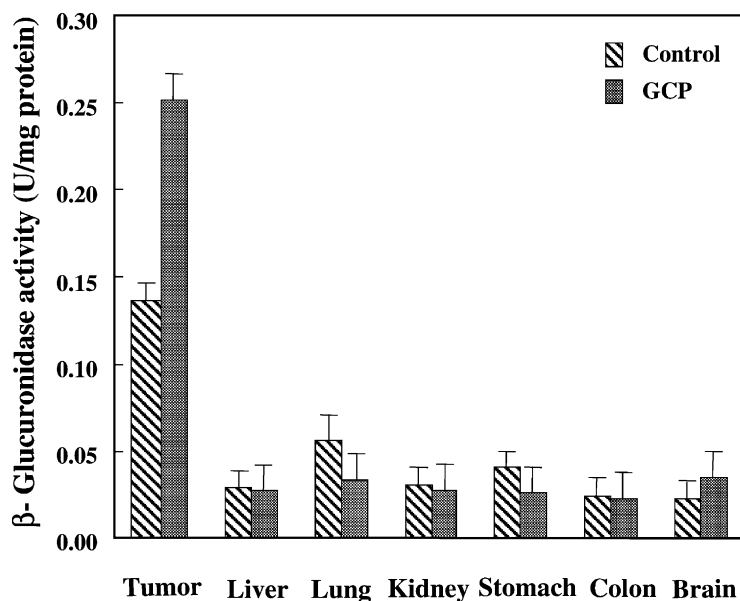


Fig. 4. β -Glucuronidase activities in tumor and non-tumor organ tissues. The tumor tissues or non-tumor were removed from control and GCPTM-treated tumor-bearing mice. The tissues were homogenized and β -glucuronidase activity was measured as described in Section 2. The β -glucuronidase activities were expressed as units per milligram protein. The data are presented as mean \pm S.D. ($n = 5$). $P_1 < 0.05$ for tumors vs. other organs in control group and $P_2 < 0.01$ for tumors vs. other organs in GCPTM-treated group.

$21.77 \pm 10.34 \mu\text{g}/\text{mg}$ protein, $P_1 < 0.01$ for tumor versus liver and $P_2 < 0.05$ for tumor versus colon). The percentages of aglycone genistein in total genistein (aglycone and conjugated) were about 80% in tumor tissues and about 20% in normal liver or colon tissues with $P_1 < 0.01$ for tumor versus liver and $P_2 < 0.05$ for tumor versus colon (Fig. 3). The level of total genistein, including aglycone and conjugated genistein, in GCPTM-treated tumor tissues was not significantly different from that in other organs. These data suggest that tumor tissues have strong biotransformation capacity for degradation of conjugated genistein into aglycone genistein. Normal organs, such as liver and colon, appear to have much weaker biotransformation capacity. Other aglycone isoflavones including daidzein and glycitein were detectable in tumors and in other organs, but their mean concentrations were lower than $1 \mu\text{g}/\text{mg}$ protein.

3.4. β -Glucuronidase activity was significantly higher in tumor tissues than in other organs

β -Glucuronidase activity was measured in tumor tissues or non-tumor organs, such as liver, lungs, kid-

neys, and brain, in both the control and GCPTM-treated groups. β -Glucuronidase activity was significantly higher in tumor tissues than in other organs for both the control ($P_1 < 0.05$) and GCPTM-treated groups ($P_2 < 0.01$) (Fig. 4). These data indicate that tumor tissue produces higher amounts of β -glucuronidase than other normal organs. As β -glucuronidase activity was significantly higher in tumor tissues of the GCPTM-treated group than those in the tumor tissues of the control group ($P < 0.01$) (Fig. 4), it is likely that genistein distributed in tumor tissues is degraded into the aglycone form more readily.

4. Discussion

Genistein has been reported to inhibit the growth of human breast cancer cell lines including both estrogen receptor positive cell lines such as MCF-7 and negative cell lines such as MDA-MB-231 in vitro and in vivo [22,23]. The mechanism of genistein-induced tumor inhibition was hypothesized to involve induction of apoptosis and G₂M arrest in breast cancer cell lines [23,24]. Genistein was found to cause cleavage

of poly(ADP-ribose)polymerase (PARP), induce the expression of p21 protein and reduce cyclin B1 expression in breast cancer cells in vitro [25–27]. Our data indicate that genistein inhibits MDA-MB-231 tumor growth in vivo by a similar mechanism.

The effect of genistein on growth of human breast cancer in xenogenic athymic mice has been controversial. Shao et al. showed an inhibitory effect of genistein on MDA-MB-231 xenogenic athymic mice. These investigators treated the tumor-bearing mice with 500 μg of genistein by oral administration [23]. In contrast, Santell et al. obtained a negative result when they treated the tumor-bearing mice with 750 μg of genistein/g diet [4]. Hsieh et al. reported that the low-dose genistein treatment (750 ppm) enhanced tumor growth in a MCF-7 tumor-bearing ovariectomized athymic model model [28]. Our results show that the treatment of MDA-MB-231 bearing mice with a relatively high dose of genistein (about 4 mg per mouse per day) resulted in statistically significant tumor regression. These findings suggest that the inhibitory effect of genistein on tumor growth in vivo may be dependent on the dose of genistein administered.

Coldham et al. reported that the biotransformation of genistein by both isolated hepatocytes and precision-cut liver slices was limited to glucuronidation of the parent compound [14]. Isoflavones circulate and are excreted in the urine mainly as glucuronide and sulfate conjugates [12,13]. Chang et al. reported genistein distribution in tissues of liver and endocrine-responsive organs such as breast, the ovary, the prostate, the testes, the thyroid and the uterus. Their studies showed that the percentage of genistein aglycone in the male liver was 34% of total genistein (aglycone and conjugation) while that in the female liver it was 77%. The percentage of genistein aglycones in male endocrine-responsive tissues was 11–45% while the percentage of female endocrine-responsive tissues was 18–100%. These results suggest that there might be a relationship between genistein glucuronide and estrogen receptor levels in liver and in endocrine-responsive organs [29].

In our recent investigation, we gave mice a high dose of GCPTM containing aglycone genistein at a concentration of 8 mg/kg body weight per day for 4 weeks. There was no adverse effect observed even

the blood concentration of genistein reached 5 $\mu\text{g}/\text{ml}$; however, this concentration of GCPTM induced substantial apoptosis in cultured tumor cells. We have also confirmed that genistein exists in blood as a form conjugated with glucuronide. The predominant existence of genistein–glucuronide as a prodrug in blood might explain why GCPTM has no apparent side effects and exerts its activity only when the conjugated glucuronide was cleaved by glucuronidase (unpublished observation).

The data presented in this study also reveal that a high degree of biotransformation of genistein aglycone correlates closely with high glucuronidase expression in tumor tissues. We also observed that in vitro cultured MDA-MB-231 cells are members of a high glucuronidase expressing cell line. The culture supernatant of the cells could convert glucoside genistin into aglycon genistein (data not shown). This indicates that the MDA-MB-231 tumor produced β -glucuronidase (heparanase) is capable of hydrolyzing isoflavone glucuronidase hydrolysis. Because there were inflammatory cells infiltrated in tumor tissues, the possibility that a part of β -glucuronidase was produced by those inflammatory cells cannot be excluded.

The important new findings from our present study are: (1) the bioactivities of genistein (aglycone) are present in higher levels in tumor tissues than in normal tissues. This might explain the reason why even high dosage of genistein administration rarely induces toxicity to normal tissues; (2) greater glucuronidase expression in tumor tissues results in greater production of genistein aglycone and, therefore, tumor tissue destruction. Because of the differential level of glucuronidase expression in different tumors, development of clinical application of genistein should be selectively considered for tumors that produce high levels of glucuronidase.

There are many components other than genistein present in GCPTM. For example, daidzein and glycitein are present and also play an important role in prevention of cardiovascular disease, brain function disorders, alcohol abuse, osteoporosis and menopausal symptoms as well as cancer prevention [30]. Other products derived from the basidiomycetes may prove to be effective agents for anti-tumor activity and immunomodulation [31]. GCPTM may exert its anti-tumor activity by inhibition of tumor proliferation and angiogenesis as well as by immunomodulation.

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