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Immunotherapy for Prostate Cancer with Gc Protein-Derived Macrophage-Activating Factor, GcMAF[1]

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Abstract

Serum Gc protein (known as vitamin D_3–binding protein) is the precursor for the principal macrophage-activating factor (MAF). The MAF precursor activity of serum Gc protein of prostate cancer patients was lost or reduced because Gc protein was deglycosylated by serum α-*N*-acetylgalactosaminidase (Nagalase) secreted from cancerous cells. Therefore, macrophages of prostate cancer patients having deglycosylated Gc protein cannot be activated, leading to immunosuppression. Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generated the most potent MAF (termed GcMAF) ever discovered, which produces no adverse effect in humans. Macrophages activated by GcMAF develop a considerable variation of receptors that recognize the abnormality in malignant cell surface and are highly tumoricidal. Sixteen nonanemic prostate cancer patients received weekly administration of 100 ng of GcMAF. As the MAF precursor activity increased, their serum Nagalase activity decreased. Because serum Nagalase activity is proportional to tumor burden, the entire time course analysis for GcMAF therapy was monitored by measuring the serum Nagalase activity. After 14 to 25 weekly administrations of GcMAF (100 ng/week), all 16 patients had very low serum Nagalase levels equivalent to those of healthy control values, indicating that these patients are tumor-free. No recurrence occurred for 7 years.

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Introduction

Prostate cancer is the most common malignancy among elderly men. Treatment of the metastatic cancer with hormone therapy temporarily controls symptoms in 70% to 80% of patients [1]. After a remission period, a relapse invariably occurs. After progression, no effective treatment is readily available, and the median survival is approximately 6 months in duration [2]. Therefore, progressive metastatic hormone-refractory disease remains a therapeutic challenge. In light of the mechanisms believed to be involved in the development of recurrent disease, vigorous effort is being focused on the identification of nonendocrine treatments [2–5]. However, therapeutic approaches capable of tumoricidal to hormone-refractory cancerous cells and improving the quality of life are limited to a certain immunotherapy without causing adverse effects.

Intratumor administration of bacille Calmette–Guérin (BCG) or other bacterial cells can result in regression of local and metastasized tumors, suggesting the development of specific immunity against the tumors [6,7]. However, administration of BCG into noncancerous normal tissues results in no significant effect on the tumors. Inflamed noncancerous normal tissues release membranous lipid metabolites, lysophosphatidylcholine (lyso-Pc) and other lysophospholipids, which efficiently activate macrophages [8–10]. Inflamed cancerous tissues also release lipid metabolites, lysoalkylphospholipids and alkylglycerols, because cancerous cell membranes contain alkylphospholipids [11–13]. Both lysoalkylphospholipids and alkylglycerols are approximately 400 times more potent macrophage-activating agents than lysophospholipids in terms of the minimal dosages required for the optimal macrophage activation [12–16]. This suggests that highly activated macrophages can kill cancerous cells and also explains why intratumor inflammation eradicates cancerous cells [14,15].

The inflammation-derived macrophage activation is the principal macrophage activation process, which requires serum Gc protein

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(known as vitamin D_3–binding protein) [17–19] and participation of B and T lymphocytes [8–10,20–24]. Gc protein carries one trisaccharide (Figure 1) consisting of *N*-acetylgalactosamine with dibranched galactose and sialic acid termini at 420 threonine residue [20–24]. This oligosaccharide is hydrolyzed by the inducible membranous β-galactosidase (Bgl_i) of inflammation-primed (or lyso-Pc–treated) B lymphocytes to yield a macrophage-proactivating factor. This is further hydrolyzed by the membranous *Neu-1* sialidase of T lymphocytes to yield MAF, the protein with *N*-acetylgalactosamine as the remaining sugar [20–24] (Figure 1*a*). Thus, Gc protein is the precursor for the principal MAF [20–24]. However, the MAF precursor activity of prostate cancer patient Gc protein is lost or reduced, because their serum Gc protein is deglycosylated by serum α-*N*-acetylgalactosaminidase (Nagalase) secreted from cancerous cells [25,26] (Figure 1*b*). Deglycosylated Gc protein cannot be converted to MAF, resulting in no macrophage activation. Macrophages are the major phagocytic and antigen-presenting cells. Because macrophage activation for phagocytosis and antigen presentation to B and T lymphocytes is the first indispensable step in the development of both humoral and cellular immunity, lack of macrophage activation leads to immunosuppression [25–30]. Advanced cancer patients have high serum Nagalase activities, resulting in no macrophage activation and severe immunosuppression that explain why cancer patients die with overwhelming infection (e.g., pneumonia) [25,26].

Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generates the most potent MAF (termed GcMAF) [20–24] (Figure 1*c*), which produces no adverse effects in humans [14,15,23,27]. Administration of 100 ng of GcMAF to humans results in the maximal activation of macrophages with 30-fold increased ingestion index and 15-fold increased superoxide-generating capacity [23] in 3.5 hours. GcMAF also has a potent mitogenic capacity to act on the myeloid progenitor cells, resulting in a 40-fold increase in systemic macrophage cell counts in 4 days [23,31]. Such highly activated systemic macrophages are chemotactically recruited to inflamed lesions by 180-fold increase of the macrophage cell counts [31]. Macrophages activated by GcMAF develop a considerable variation of receptors that recognize the abnormality in cancerous cell surface and kill cancerous cells [14,15,32–34]. All malignant cells have membrane abnormalities in their cell surface. A series of glycolipid, glycoprotein, and mucin antigens have been identified and designated as tumor-associated antigens on the cell surface of a wide variety of human tumor cells [35]. When human macrophages were treated *in vitro* with 100 pg GcMAF/ml for 3 hours and a prostate cancer cell line LNCaP was added with an effector/target ratio of

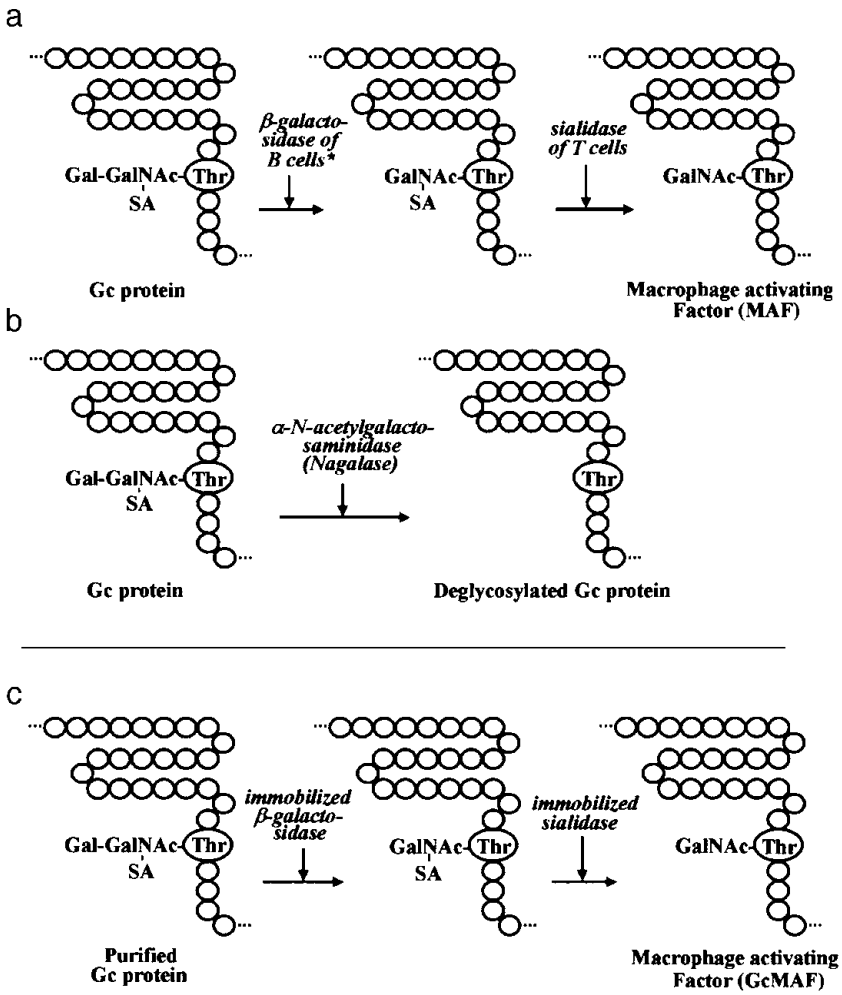

Figure 1. Schematic illustration of the formation of MAF (a), deglycosylation of Gc protein (b), and stepwise treatment of Gc protein with immobilized β-galactosidase and sialidase to generate GcMAF (c). *Asterisk* (*) indicates inflammation-primed B cells: B cells can be treated with an inflamed membranous lipid metabolite, e.g., lysophosphatidylcholine.

1.5, approximately 51% and 82% of LNCaP cells were killed by 4 and 18 hours of incubation, respectively [14,15]. This *in vitro* tumoricidal capacity of macrophages activated by GcMAF led us to investigate the therapeutic efficacy of GcMAF for prostate cancer. GcMAF therapy as a single remedy modality can eradicate metastatic breast and colorectal cancers most effectively [34,36]. Although, in recent years, prostate-specific antigen (PSA) has been used as a diagnostic and prognostic index for prostate cancer [37,38], more precision of prognostic index is desirable for therapeutic efficacy of GcMAF for prostate cancer patients. Because the serum Nagalase activity of cancer patients is directly proportional to tumor burden [25,26,32,33], serum Nagalase activity has been effectively used as a diagnostic index for a variety of cancers [14,15,25,26,32,33,39] and as a prognostic index for radiation therapy [25], surgical resection of tumors [26], and GcMAF therapy for preclinical and clinical mammary adenocarcinoma models [32–34] and colorectal cancers [36].

Materials and Methods

Chemicals and Reagents

Phosphate-buffered saline (PBS) contained 1 mM sodium phosphate and 0.15 M NaCl. When peripheral blood monocytes adhere to the vessel substratum, they behave like macrophages that show increased synthesis of hydrolases. For manipulation *in vitro* and cultivation of peripheral blood mononuclear cells containing monocytes/macrophages (macrophages for short) and lymphocytes (B and T cells), 0.1% egg albumin–supplemented RPMI-1640 medium (EA medium) was used. Sera for isolation of Gc1 protein (major Gc isoform) were donated by members of the institute and were routinely screened to be virus-free using ELISA assays for antibodies against human immunodeficiency and hepatitis B and C viruses (Cambridge Biotechnology, Cambridge, UK, and Abbott Laboratories, Abbot Park, IL). Gc protein was purified by vitamin D–affinity chromatography [23,40]. β-Galactosidase and sialidase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, and were immobilized on Sepharose [21–23]. Lysophosphatidylcholine (lyso-Pc) and *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide were purchased from Sigma Chemical Co. (St. Louis, MO).

Procedure for Preparation of GcMAF

Serum was heat-inactivated at 60°C for 1 hour and was mixed with 30% saturated ammonium sulfate that precipitates Gc protein fraction [41]. The precipitate was dissolved in PBS (pH 7.4) containing 0.5% Triton X-100 and 0.3% tri-*n*-butyl phosphate and was kept overnight at room temperature to resolve the lipid containing microbial contaminants, including enveloped viruses, if any. The samples were precipitated by 30% saturated ammonium sulfate, dissolved in citrate buffer at pH 4.0, and kept overnight. Gc protein was purified using 25-hydroxyvitamin D_3–affinity chromatography [40]. This chromatographic specificity to Gc protein yields highly pure Gc protein and eliminates all possible contaminations of macromolecules. Electrophoretic analysis proved the purity of Gc protein (MW 52,000). Stepwise incubation of the purified Gc protein with immobilized β-galactosidase and sialidase yielded probably the most potent MAF (GcMAF) ever discovered [21–23] (Figure 1*c*). The immobilized enzymes were removed by centrifugation. Thus, GcMAF is pure and free from contamination of the enzymes. The final product, GcMAF, was filtered through a low protein-binding filter, Millex-HV (Millipore Corp., Bedford, MA) for sterilization.

Because the molecular structure of GcMAF is identical to that of native human MAF (Figure 1, *a* and *c*), it should have no adverse effects on humans. In fact, numerous administrations (more than 10 times for 3- to 6-month period) of GcMAF (100–500 ng/human) to 12 humans showed no signs of adverse effects [15,23]. The optimal human dose of GcMAF, to achieve phagocytic capacity by 30-fold increased ingestion index and 15-fold increased superoxide-generating capacity of peripheral blood monocytes/macrophages, was found to be approximately 100 ng/human. Quality control of preparation of GcMAF was performed for activity, sterility, and safety tests.

GcMAF Therapy for Prostate Cancer Patients

Participants. A group of 16 nonanemic prostate cancer patients was included in this study. Although serum Nagalase activities of prostatectomized patients indicate significant amounts of metastasized tumor cells, computed tomography did not detect metastasized tumor lesions in other organs. These patients received GcMAF therapy exclusively and excluding combination therapy with erythropoiesis induction. Thus, anemic prostate cancer patients were not eligible in the program. The study was approved by the institutional research and ethic committees of Nagasaki Immunotherapy Group, Nagasaki, Japan, and by the institutional review board of Hyogo Immunotherapy Group, Hyogo, Japan. The participants gave written informed consent before entering the study.

GcMAF administration. Because the half-life of the activated macrophages is approximately 6 days [12,13], 100 ng of GcMAF was administered intramuscularly once a week.

Procedures to be used for clinical study and study parameters. Serum samples (>2 ml) were weekly or biweekly collected immediately before each GcMAF administration and were used for prognostic analysis. Detailed assessment of patient response to each GcMAF administration was performed by determining both MAF precursor activity of serum Gc protein and serum Nagalase activity. Because serum Nagalase activity is proportional to tumor burden [26,32,33], kinetic assessment of curative response to GcMAF therapy was performed by determining serum Nagalase activity as a prognostic index during the entire therapeutic course of all 16 patients. The PSA values were also determined immediately before this study.

Assay for MAF Precursor Activity of Patient Serum Gc Protein

Blood samples of healthy humans were collected in tubes containing EDTA to prevent coagulation. A 5-ml blood sample and 5 ml of saline (0.9% NaCl) were mixed and gently laid on a 15-ml centrifuge tube containing 3 ml of Lymphoprep (similar to Ficoll; Polysciences, Inc, Warrington, PA) and centrifuged at 800*g* for 15 minutes. The dense white cell band as peripheral blood mononuclear cells containing monocytes/macrophages (macrophages for short) and lymphocytes (B and T cells) was collected using a Pasteur pipette. The white cell mixture was washed twice with PBS, suspended in EA medium, and placed in 16-mm wells. Incubation for 45 minutes in a 5% CO_2 incubator at 37°C allowed adherence of macrophages to the plastic surface. The mixture of lymphocytes and adherent macrophages of healthy humans was treated with 1 μg lyso-Pc/ml in EA medium for 30 minutes. Because of the adherence of macrophages to the plastic substrata, lymphocytes and macrophages were separately washed with PBS, admixed, and cultured in EA medium containing

0.1% serum of prostate cancer patients or healthy human as a source of Gc protein. After 3 hours of cultivation, the macrophages were assayed for superoxide-generating capacity [25,26]. The macrophages were washed with PBS and incubated in 1 ml of PBS containing 20 μg of cytochrome *c* for 10 minutes. Thirty minutes after the addition of phorbol-12-myristate acetate (5 μg/ml), the superoxide-generating capacity of the macrophages was determined spectrophotometrically at 550 nm. The data were expressed as nanomoles of superoxide produced per minute per 10^6 cells (macrophages). These values represent the MAF precursor activity of patient serum Gc protein [28,29]. Lost or reduced MAF precursor activity of patient serum Gc protein is expressed as a decrease in superoxide generation compared with the healthy human Gc protein control. Thus, the MAF precursor activity measures both the ability of each patient to activate macrophages and the immune potential. However, loss of MAF precursor activity results in immunosuppression.

Cultivation of the mixture of lyso-Pc–treated lymphocytes and macrophages in EA medium without serum results in the production of 0.5 to 0.85 nmol superoxide/min per 10^6 cells [41,42]. Thus, if patient serum (0.1%) generates <0.85 nmol superoxide/min per 10^6 cells, the precursor activity of patient serum Gc is considered to be lost.

Determination of Nagalase Activity in Patient Blood Stream

Patient sera (300 μl) were precipitated with 70% saturated ammonium sulfate. The precipitates were dissolved in 50 mM sodium citrate buffer (pH 6.0) and were dialyzed against the same buffer at 4°C for 2 hours. The dialysates were made up to 1 ml in volume and assayed for Nagalase activity [25,26]. Substrate solution (250 μl) contained 5 μmol of *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide in 50 mM citrate buffer (pH 6.0). The reaction was initiated by the addition of 250 μl of the dialyzed samples, kept at 37°C for 60 minutes, and terminated by adding 200 μl of 10% TCA. After centrifuging the reaction mixture, 300 μl of 0.5 M Na_2CO_3 solution was added to the supernatant. The amount of released *p*-nitrophenol was determined spectrophotometrically at 420 nm and was expressed as nanomoles per minute per milligram protein [25,26]. Protein concentrations were estimated by the Bradford method [43].

The half-life of Nagalase activity *in vivo* is less than 24 hours, because we observed a sudden drop of Nagalase activity in 24 hours after resection of the tumor [26]. However, Nagalase activity in the collected serum is extremely stable, probably because of the presence of a product inhibitor, and is highly reproducible after storage of sera at 4°C for more than 6 months [26].

Healthy control sera exhibit low levels (0.35–0.65 nmol/min per milligram) of the enzyme activity. This is the enzyme activity of α-galactosidase that can catabolize the chromogenic substrate (i.e., *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide) for Nagalase [25,26,28]. The reduction in serum Nagalase activity to 0.65 nmol/min per milligram or less in patients during GcMAF therapy serves as demonstration that tumor burden has been eradicated.

Results

Therapeutic History and Immunodiagnostic Parameters of Nonanemic Prostate Cancer Patients

The therapeutic history of 16 prostate cancer patients before GcMAF therapy is summarized in Table 1. Nine patients received prostatectomy with or without hormone therapy. A total of 12 patients received hormone therapy. Because the fate and staging of the malignant disease correlate with tumor burden and the degree of immunosuppression [25,26], the immune potency and tumor burden index for each patient must be determined before entering GcMAF therapy regardless of the lapse of time after prostatectomy and/or hormone therapy (Table 1).

Table 1. Therapeutic History and Diagnostic Parameters of Prostate Cancer Patients.

Patient			Therapeutic History			Pre–GcMAF Therapy*		
No.	Age (years)	PSA	Surgery	PSA	Endocrine	PSA	Precursor	Nagalase
1	64	16.5	No	—	Yes	21.7	0.82	4.92
2	76	2.5	Pxy	<0.1	None	3.52	3.19	2.30
3	46	35.4	Pxy	0.3	Yes	8.2	2.44	2.85
4	68	4.5	No	—	Yes	4.2	2.26	3.25
5	68	68.4	Pxy	0.2	Yes	0.09	3.77	3.15
6	50	20.5	Pxy	0.1	Yes	1.0	2.20	3.73
7	56	18.0	Pxy	0.2	Yes	3.4	2.88	1.95
8	61	25.3	Pxy	0.2	None	5.8	2.29	3.45
9	56	8.4	No	—	Yes	6.5	2.85	2.50
10	53	8.0	Pxy	0.1	None	3.8	0.85	4.72
11	66	16.6	No	—	Yes	10.2	2.05	4.02
12	66	22.5	Pxy	0.1	Yes	4.2	2.75	3.62
13	68	6.2	No	—	Yes	10.1	1.02	5.34
14	73	3.1	No	—	Yes	3.2	2.23	3.52
15	58	6.0	Pxy	0.1	None	7.8	1.68	4.32
16	63	6.6	No	—	Yes	5.8	2.22	3.58
C†							4.84	0.39‡

Pxy indicates prostatectomy.
*Pre–GcMAF therapy assays for PSA, precursor activity (nmol/min per 10^6 cells), and Nagalase (nmol/min per milligram). The precursor activity of <0.9 nmol/min per 10^6 cells is unable to develop phagocytic capacity of macrophages and is considered to be loss of the precursor activity.
†Average of seven healthy controls.
‡This activity level is the enzyme activity of α-galactosidase and not of Nagalase.

Because macrophage activation for phagocytosis and antigen presentation to B and T cells is the first indispensable step for immune development, the lack of macrophage activation leads to immunosuppression [26,27]. Because serum Gc protein is the precursor of the principal MAF, the MAF precursor activity of patient serum Gc protein was first to be determined. As shown in Table 1, the MAF precursor activities of serum Gc protein of prostate cancer patients were lost (<0.85 nmol superoxide/min per 10^6 cells) or reduced. Because loss or decrease in the MAF precursor activity of patient Gc protein results from deglycosylation of the Gc protein by serum Nagalase secreted from cancerous cells [25,26] (Figure 1*b*), serum Nagalase activities of these cancer patients were determined. Patients having lower precursor activity of the Gc protein carried higher serum Nagalase activity (Table 1). Because serum Nagalase activity of cancer patients is directly proportional to their tumor burden [26,32,33], the serum Nagalase activity indicates the total amount of the primary tumor (if not prostatectomized) and the metastasized tumor cells. Thus, the serum Nagalase activity of individual patients should be used as a baseline control for prognostic analysis during GcMAF therapy. The PSA values of each patient at the initial diagnosis, after prostatectomy, and before entering GcMAF therapy are also shown in Table 1.

The MAF Precursor Activity of Gc Protein and Serum Nagalase Activity as Prognostic Parameters during GcMAF Therapy for Prostate Cancer Patients

In the course of GcMAF therapy, MAF precursor activity and serum Nagalase activity of five patients were analyzed. As GcMAF therapy progressed the MAF precursor activity of all five patients increased and their serum Nagalase activity decreased inversely as shown in Table 2. To illustrate quantitative correlation of these parameters,

Table 2. Correlation of the MAF Precursor Activity of Individual Prostate Cancer Patients with Their Serum Nagalase Activity during GcMAF Therapy.

Patient No.	Time Assayed (weeks)	Precursor Activity Superoxide (nmol)	Nagalase (nmol/min per milligram)
1 (7)*	0	2.88	1.95
	1	3.38	1.74
	2	3.51	1.59
	4	3.61	1.32
	6	3.44	1.19
	10	3.92	1.08
	12	4.05	0.96
	21	4.13	0.68
2 (8)	0	2.29	3.45
	1	2.40	2.89
	2	2.62	2.75
	3	2.88	2.43
	4	2.92	2.21
	6	3.21	2.02
	10	3.33	1.69
	14	3.62	1.38
	17	3.72	0.94
	21	4.29	0.66
3 (6)	0	2.20	3.73
	1	2.28	3.09
	2	2.75	2.73
	4	3.18	3.34
	8	3.03	2.18
	11	3.33	2.01
	15	3.50	1.89
	19	3.65	1.67
	23	3.75	1.29
	26	4.24	0.67
4 (12)	0	2.75	2.62
	1	3.11	3.16
	2	3.16	2.01
	3	3.20	1.82
	9	3.25	1.71
	16	3.35	1.43
	22	4.23	0.64
5 (13)	0	1.02	5.34
	1	1.22	5.11
	2	1.37	4.87
	3	1.68	4.31
	4	1.97	4.12
	9	2.22	3.54
	14	3.81	1.10
	18	3.79	0.83
	24	4.28	0.72
Control†		4.25	0.52

*The numbers in parentheses refer to the patient nos. in Table 1.
†The mean value of five healthy controls.

the time course of MAF precursor activity of individual prostate cancer patient was plotted against their corresponding serum Nagalase activity. As GcMAF therapy progressed, the MAF precursor activity increased with a concomitant decrease in serum Nagalase activity as shown in Figure 2. These prognostic parameters of all five individual patients fall in the same linear inverse correlation. When the MAF precursor activity increased toward the healthy control value, serum Nagalase activities of these patients decreased toward the healthy control level (Figure 2). Thus, these malignancy parameters of prostate cancer patients served as excellent prognostic indices. Because the serum Nagalase is proportional to tumor burden [26,32,33], as GcMAF therapy progressed, serum Nagalase activity decreased and, concomitantly, tumor burden decreased. Thus, the entire time course analysis of tumor burden during GcMAF therapy of all 16 patients should be performed by measuring serum Nagalase activity as a prognostic index. The kinetic decrease of serum Nagalase activity allows us to envision a curative process of the malignancy as a decrease of tumor burden.

Time Course Study of Serum Nagalase Activity of Prostate Cancer Patients during GcMAF Therapy

Time course analyses of serum Nagalase activity of the prostate cancer patients assess the efficacy of GcMAF. These patients had the initial Nagalase activities ranging from 1.95 to 5.34 nmol/min per milligram (Table 1). As shown in Figure 3, the serum Nagalase activities of all 16 patients decreased as GcMAF therapy progressed. After approximately 14 to 25 administrations (14–25 weeks) of 100 ng of GcMAF, all 16 patients had very low serum Nagalase activity levels equivalent to those of healthy control values ranging from 0.37 to 0.68 nmol/min per milligram. These low enzyme activities are those of α-galactosidase and not of malignant-specific Nagalase [25,26]. Because serum Nagalase activity is proportional to tumor burden, the results suggest that these patients are free of cancerous cells. During 7 years of observation after completion of GcMAF therapy, these patients showed no increase in their serum Nagalase activities, indicating no recurrence of prostate cancer. Furthermore, annual computed tomographic scans of these patients confirmed them being tumor recurrence–free for the 7 years.

Curative Rate of GcMAF Therapy for Prostate Cancer Depends on the Degree of Cell Surface Abnormality

Poorly differentiated (termed undifferentiated) cancer cells should have more abnormality in cell surface than moderately/immediate differentiated (termed differentiated for short) cancer cells [34,36]. Because the activated macrophages efficiently recognize and rapidly kill cancer cells having more abnormality, the activated macrophages kill undifferentiated cells more rapidly than differentiated cells [34,36]. Thus, the rapidly decreasing serum Nagalase activities during GcMAF therapy imply more abnormality in undifferentiated cells. As shown in the time course study of GcMAF therapy in Figure 3, the serum Nagalase activity of patient nos. 2, 5, 7, 8, 9, 11, 12, and 16, for example, decreased sharply in the first few weeks (up to 4 weeks), followed by a slow decrease during the remaining therapeutic period (approximately 8–14 weeks). These biphasic tumor regression graphs suggest that undifferentiated cells are mixed with differentiated cells

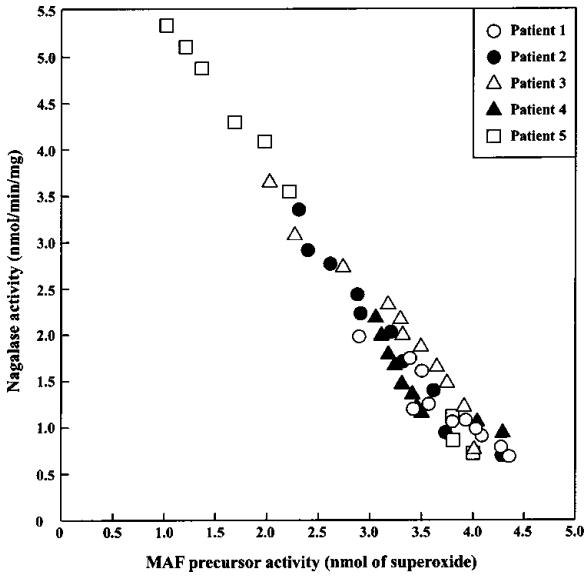

Figure 2. Inverse correlation between the MAF precursor activity of serum Gc protein and serum α-*N*-acetylgalactosaminidase (Nagalase) activity of prostate cancer patients during GcMAF therapy.

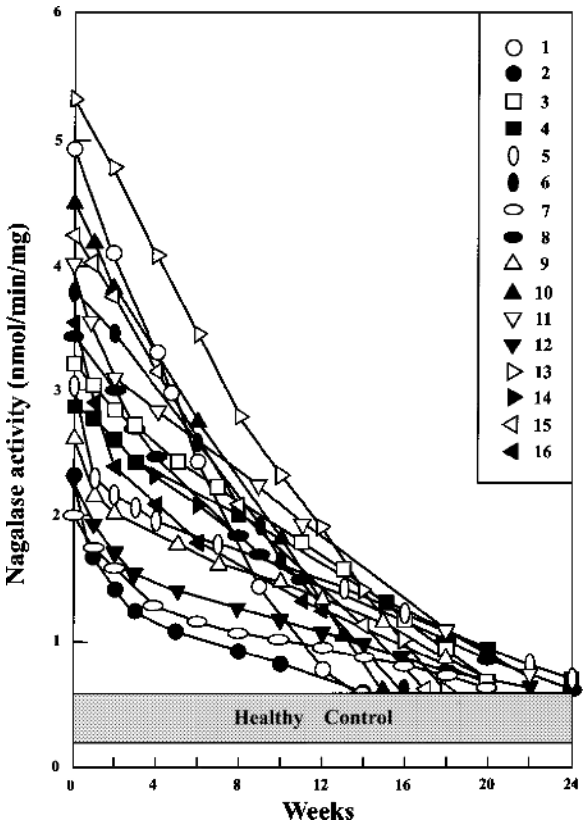

Figure 3. Time course study of GcMAF therapy of 16 prostate cancer patients with serum α-*N*-acetylgalactosaminidase (Nagalase) as a prognostic index.

within the tumors [34,36]. Thus, undifferentiated cells were killed rapidly during the first few weeks, and the differentiated cells were killed slowly in the remaining GcMAF therapeutic period. These mixed-cell populations seemed to be developed by differentiation during the growth of undifferentiated tumor cells [34,36]. In contrast, patient nos. 1, 6, 10, 13, and 15 showed that their serum Nagalase activity decreased linearly and rapidly reached control values between 14 and 18 weeks. These linear, declining curative rates are slower than the curative rates of the undifferentiated population of the previously mentioned mixed population (i.e., undifferentiated and differentiated) of prostate cancers. Therefore, the latter group of tumors is already differentiated and further differentiation of these tumors did not occur during tumor growth. Similar results were also observed during GcMAF therapy for metastatic breast cancer patients [34].

Correlation between Serum Nagalase Activity and PSA Levels during GcMAF Therapy

Because serum Nagalase activity is an excellent index for the estimation of tumor burden [26,32], serum PSA levels were compared with serum Nagalase activity during GcMAF therapy of five prostate cancer patients. As shown in Table 3, PSA levels of prostatectomized patients decreased as serum Nagalase decreased during GcMAF therapy. In patients without tumor resection, however, although serum Nagalase activity decreased as GcMAF therapy progressed, their PSA values remained unchanged. The result suggests that the PSA derived from tumor-bearing prostate did not change while tumor burden decreased. Because tumor-induced inflammation in the noncancerous prostate tissues causes secretion of PSA [38], the PSA produced from these inflamed noncancerous prostate tissues cannot be changed by the decrease in tumor burden.

Discussion

Prostatic cancer diagnosis and prognosis have been aided by the availability of PSA measurement [37,38]. When patients received radical prostatectomy, a sudden drop of high PSA levels to very low values was observed (Table 1). Thus, PSA is predominantly produced from primary tumor lesions in prostate compared with the metastasized lesions. Although serum Nagalase decreased during GcMAF therapy of patients with tumor-bearing prostate, PSA remained unchanged (Table 3). Therefore, PSA values cannot be used for prognostic assays during GcMAF therapy.

Prostate-specific antigen, as a serine protease, has been considered to be specific to prostatic malignancy and one of extracellular matrix–degrading enzymes that is required for invasiveness of cancerous tissues [44,45]. However, normal prostatic inflamed tissue can release PSA [38], particularly in certain disease states such as benign prostate hypertrophy and prostatitis. The invasive enzymatic action of the cancerous PSA onto surrounding noncancerous prostatic tissues induces a mild inflammatory process that can cause noncancerous prostate cells to release PSA. Because of the predominance of PSA production in prostatic tissues, PSA assay cannot accurately estimate the fractional loss of tumor burden. This is confirmed in the present

Table 3. Correlation between Serum Nagalase Activity and PSA during Time Course Study of GcMAF Therapy for Prostate Cancer Patients.

Patient No.	Age (years)	Assayed Weeks After first GcMAF	Nagalase Specific Activity (nmol/min per milligram)	PSA (ng/ml)
A1	67	0	2.53	27.60
		2	2.27	27.20
		4	2.00	25.90
		5	1.94	28.25
		6	1.93	23.79
		12	1.47	26.74
		24	0.69	25.95
A2	83	0	3.66	18.02
		2	2.94	15.64
		3	2.74	13.91
		4	2.63	18.77
		5	2.55	21.94
		10	0.72	18.53
A3	60	0	2.18	58.49
		3	1.85	42.49
		4	1.77	56.54
		5	1.62	63.61
		6	1.62	82.30
		7	1.54	65.20
		10	0.84	58.45
A4 (prostatectomy)	76	0	3.94	11.85
		1	3.44	10.56
		4	2.46	5.22
		12	1.92	0.33
		15	1.36	0.24
		20	0.69	0.10
A5 (prostatectomy)	66	0	2.00	5.82
		1	1.79	5.43
		2	1.69	4.05
		4	1.49	3.12
		6	1.38	2.77
		9	1.21	2.46
		13	1.19	1.89
		18	1.07	0.86
		21	0.92	0.14
		26	0.62	0.10

article by comparative analysis of serum Nagalase activity with PSA during GcMAF therapy (Table 3).

Furthermore, PSA, being a serine protease, may not be restricted to the prostate. Prostate-specific antigen has been shown to be produced by extraprostatic tissues, including salivary gland neoplasm, cloacogenic glandular epithelium, and normal and cancerous female breast tissues [46–48]. Thus, PSA is less specific to prostatic malignancy.

In contrast, Nagalase is secreted exclusively from cancerous cells but not from normal tissues (even inflamed noncancerous tissues). Thus, the level of Nagalase activity in blood stream is proportional to the tumor burden in the hosts [25,28,29] and has been used as a prognostic index for GcMAF therapy for preclinical and clinical cancer models [14,15,25,32–34,36]. Serum Nagalase deglycosylates serum Gc protein. Deglycosylated Gc protein loses its MAF precursor activity and cannot be converted to MAF, resulting in no macrophage activation leading to immunosuppression [25,26]. Thus, measurement of serum Nagalase activity and the MAF precursor activity of serum Gc protein allows us to envision the degree of immunosuppression and the state of the disease.

Because the trisaccharide of Gc protein in the blood stream is efficiently deglycosylated by serum Nagalase [25–28] (Figure 1*b*), the serum Nagalase seems to be an endo-Nagalase but not as an exo-enzyme under colloidal serum environment. When GcMAF (100 ng) is administered to cancer patients, GcMAF is not affected by the patient's serum Nagalase [34,36], bypasses the deglycosylated Gc protein, and directly acts on macrophages for an extensive activation. Such highly activated macrophages develop a considerable variation of receptors, recognize the abnormality of malignant cell surface, and eradicate cancerous cells [34,36]. This fundamental nature of macrophages to recognize abnormality of malignant cells is universal to all types of cancers. In fact, administration of GcMAF (100 ng/week) to nonanemic cancer patients showed curative effects on a variety of cancers indiscriminately [15,33,34,36]. The types of cancer so far tested are prostate, breast, colon, stomach, liver, lung (including mesothelioma), kidney, bladder, uterus, ovary, head/neck, melanoma, and fibrosarcoma [34]. Progress of GcMAF therapy for these cancers is monitored by the measurement of the malignant cell–specific serum Nagalase activity that is universally found in patients with a wide variety of cancers [25,26]. Curative rates of various cancers depend on the degree of cell surface abnormality that corresponds to the grade of differentiation of the malignant cells. Precision of measurement of Nagalase activity allowed us to determine the degree of cell surface abnormality by the curative rate during GcMAF therapy. Undifferentiated tumor cells are killed more efficiently than differentiated cells [34,36]. In fact, adenocarcinomas such as breast and prostate cancer cells are undifferentiated and killed rapidly by the activated macrophages, whereas well-differentiated cancer cells such as squamous carcinoma cells are slowly killed by the activated macrophages. Faster curative rates, requiring less than 22 weeks, were always observed during GcMAF therapy for breast cancer [34]. In contrast, GcMAF therapy for well-differentiated squamous cell carcinomas such as head/neck cancers requires more than 75 weeks. Thus, the faster curative rate of prostate cancer is due to the efficient macrophage recognition of the abnormality of the prostate cancer cell surface. However, a variety of cancers contain the mixed population of undifferentiated and differentiated cells within a tumor (e.g., breast and colorectal cancers) [34,36]. This type of fine differentiation in prostate cancer has been known for many years. In 1977, Gleason [49] separated histologic patterns of prostate cancer into a grading 1 to 5 patterns of decreasing differentiation, tumor pattern grade 1 being most differentiated and pattern grade 5 being least differentiated (poorly differentiated or undifferentiated). Tumor pattern grade 3 (Gleason grade 3) is the most common histologic pattern and is considered moderately well differentiated. However, one can readily interpret the histologic pattern of the grade 3 (schematic diagram developed by Gleason) as a mixture of differentiated cells (grade 1) and least differentiated (undifferentiated) cells (grade 5). This can explain the biphasic tumor regression graphs during GcMAF therapy for most prostate cancer being a mixture of differentiated and undifferentiated cells.

Because of the availability of precision measurement of serum Nagalase, the curative rate measurements of tumors during GcMAF therapy and the estimation of the degree of tumor differentiation have been possible. Therefore, the significance of GcMAF therapy for cancers has been greatly enhanced by the discovery of cancer cell–specific Nagalase that can accurately monitor the rate of tumor regression during GcMAF therapy [32–34,36].

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