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Immunotherapy of metastatic breast cancer patients with vitamin D-binding protein-derived macrophage activating factor (GcMAF)

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Serum vitamin D_3-binding protein (Gc protein) is the precursor for the principal macrophage activating factor (MAF). The MAF precursor activity of serum Gc protein of breast cancer patients was lost or reduced because Gc protein was deglycosylated by serum α-*N*-acetylgalactosaminidase (Nagalase) secreted from cancerous cells. Patient serum Nagalase activity is proportional to tumor burden. The deglycosylated Gc protein cannot be converted to MAF, resulting in no macrophage activation and immunosuppression. Stepwise incubation of purified Gc protein with immobilized β-galactosidase and sialidase generated probably the most potent macrophage activating factor (termed GcMAF) ever discovered, which produces no adverse effect in humans. Macrophages treated *in vitro* with GcMAF (100 pg/ml) are highly tumoricidal to mammary adenocarcinomas. Efficacy of GcMAF for treatment of metastatic breast cancer was investigated with 16 nonanemic patients who received weekly administration of GcMAF (100 ng). As GcMAF therapy progresses, the MAF precursor activity of patient Gc protein increased with a concomitant decrease in serum Nagalase. Because of proportionality of serum Nagalase activity to tumor burden, the time course progress of GcMAF therapy was assessed by serum Nagalase activity as a prognostic index. These patients had the initial Nagalase activities ranging from 2.32 to 6.28 nmole/min/mg protein. After about 16–22 administrations (approximately 3.5–5 months) of GcMAF, these patients had significantly low serum enzyme levels equivalent to healthy control enzyme levels, ranging from 0.38 to 0.63 nmole/min/mg protein, indicating eradication of the tumors. This therapeutic procedure resulted in no recurrence for more than 4 years.

Key words: macrophage activating factor; tumoricidal; immunotherapy; deglycosylation; α-*N*-acetylgalactosaminidase

The number of elderly breast cancer patients has increased significantly in the last few decades.[1,2] It has been reported that more than 40% of newly-diagnosed breast cancer occurs in women age older than 65 years and most older women present more frequently with advanced disease.[3] At least 50% of the patients die as a consequence of metastatic disease. Even in node-negative breast cancer patients one third will have distant recurrence within several years.[4] Metastatic breast cancer patients are essentially incurable with standard therapy, and have a median survival duration of less than 2 years after documentation of metastases.[4,5] While sensitive to initial treatment such as hormonal therapy, metastatic breast cancer virtually always progresses and responses are harder to obtain with subsequent regimens. Therapeutic approaches capable of tumoricidal to hormone-refractory and chemoresistant cancerous cells are limited to a certain immunotherapy without producing side effects.

Inflammation of cancerous tissues induced by intratumor administration of BCG (*Bacille Calmette Guerin*) or other bacterial cells can result in regression of local as well as metastasized tumors, suggesting development of specific immunity against the tumors.[6,7] However, inflammation in noncancerous normal tissues results in no significant effect on the tumors.[7] Inflamed normal tissues release membranous lipid metabolites, lyso-phosphatidylcholine (lyso-Pc) and other lysophospholipids, that efficiently activate macrophages.[8–11] Inflamed cancerous tissues release lipid metabolites, lysoalkylphospholipids and alkylglycerols, because cancerous tissues contain alkylphospholipids.[11–14] Both lysoalkylphospholipids and alkylglycerols are at least 400 times more potent macrophage activating agents than lysophospholipids in terms of the minimal dosages required for optimal macrophage activation.[12–14] These observations suggest that highly activated macrophages are tumoricidal and also explain why inflammation in cancerous tissues cures the disease. In fact, highly activated macrophages have a potential to kill and eradicate cancer cells.[15–18]

Inflammation-derived macrophage activation is the principal macrophage activation process which requires serum vitamin D binding protein (known as Gc protein)[19–24] and participation of B and T lymphocytes.[9–14] Gc protein carries a trisaccharide composed of *N*-acetylgalactosamine with dibranched galactose and sialic acid terminals.[25] This oligosaccharide is hydrolyzed by the inducible membranous β-galactosidase (Bgl_i) of inflammation-primed (or lysoPc-treated) B cells to yield a macrophage proactivating factor. This in turn is hydrolyzed by the membranous *Neu-1* sialidase of T-cells to yield the macrophage activating factor (MAF)[22–27] (Fig. 1*a*). Thus, Gc protein is the precursor for the principal MAF.[22,27] However, the MAF precursor activity of 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[28–31] (Fig. 1*b*). Thus, serum Nagalase activity is proportional to tumor burden.[29,32] Deglycosylated Gc protein cannot be converted to MAF, resulting in no macrophage activation. Since macrophage activation for phagocytosis and antigen presentation to B and T lymphocytes is the first indispensable step in humoral and cellular immunity development, lack of macrophage activation leads to immunosuppression.[28–31,33–36] Advanced cancer patient sera contain high Nagalase activity with a concomitant loss of the MAF precursor activity. Thus, lack of macrophage activation and severe immunosuppression in advanced cancer patients explain why they die with overwhelming infection (*e.g.*, pneumonia).[29]

Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generates probably the most potent MAF (termed GcMAF)[23–27,32,37–39] ever discovered (Fig. 2), which produces no side effect in humans.[30,32,38,40] Administration of 100 ng GcMAF per human results in the maximal level of macrophage activation which develop an enormous variation of receptors that recognize abnormality in malignant cell surface and kill cancerous cells.[30,32,37,38] All malignant cells have abnormalities in their cell surface. A series of glycolipid, glycoprotein and mucin antigens have been identified and designated as tumor-associated antigen (TAA) on the cell surface of a wide variety of tumor cells.[41–44] When human macrophages were treated *in vitro* with GcMAF (100 pg/ml) for 3 hr and a breast cancer cell line MCF-7 was added with effector/target ratio of 1.5, 60% and 86% of MCF-7 cells were killed in 4 hr and 18 hr incubation, respectively.[38,40]

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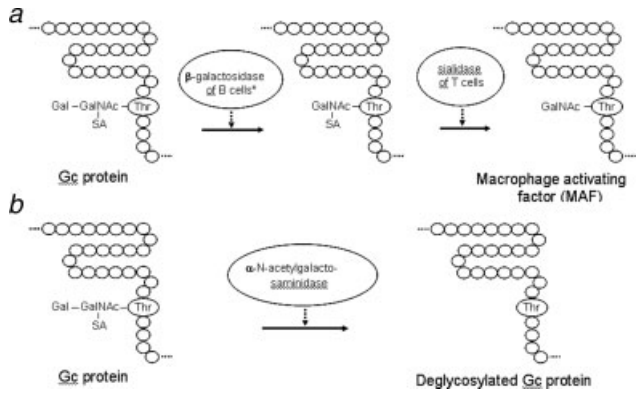

Figure 1 – Schematic illustration of formation of MAF (*a*) and deglycosylation of Gc protein (*b*). *, inflammation-primed B cells: B cells can be treated with an inflamed membranous lipid metabolite, *e.g.*, lysophosphatidylcholine.

When mice bearing a mammary adenocarcinoma (Ehrlich ascites tumor) were treated with 4 administrations of 100 pg GcMAF, serum Nagalase activity decreased to the enzyme activity level of control healthy mice.[32,37] These mice still carried a trace amount of ascites cells.[37] We designated them as dormant cells because they were nonproliferating. Later we found that they are terminally differentiated osteoblast cells. Thus, GcMAF therapy eradicates Ehrlich ascites tumor cells.

Although in recent years CA27.29, CA15-3 and carcinoembryonic antigen (CEA) have been diagnostic and prognostic indices for breast cancer,[45,46] more precision of prognostic index is desirable for therapeutic efficacy of GcMAF for breast cancer patients. MAF precursor activity and serum Nagalase activity have been used as diagnostic indices for a variety of cancer patients[28–32,37,39] and as prognostic indices during radiation therapy,[47,48] surgical resection of tumors[29] and GcMAF therapy of tumor bearing mice.[32,37,47]

In this communication, therapeutic effect of GcMAF on 16 nonanemic breast cancer patients was studied to evaluate the efficacy of GcMAF for metastatic mammary adenocarcinoma. The prognostic significance for the MAF precursor activity and serum Nagalase activity of breast cancer patients is reported.

Material 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 vessel substrates they behave like macrophages which show increased synthesis of hydrolases. For manipulation *in vitro* and cultivation of peripheral blood mononuclear cells (PBMCs) containing adhering monocytes (termed macrophages) and lymphocytes (B and T cells), medium RPMI-1640 supplemented with 0.1% egg albumin (EA medium) was used. Sera for isolation of Gc1 protein (major Gc isoform) were donated by members of the institute and routinely screened for free of viruses using ELISA assays for antibodies against HIV, hepatitis B and C viruses (Cambridge Biotech and Abbott Laboratories). Human Gc1 protein was purified by vitamin D-affinity chromatography.[39,49] β-Galactosidase and sialidase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. and immobilized on Sepharose.[23–25] Lysophosphatidylcholine (lyso-Pc) and *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide were purchased from Sigma Chemical (St. Louis, MO). Using the *Limulus* amebocyte lysate assay,[7] we routinely tested for freedom of lipopolysaccharide contamination in the stock solution of enzyme suspension, Gc protein, GcMAF and culture media.

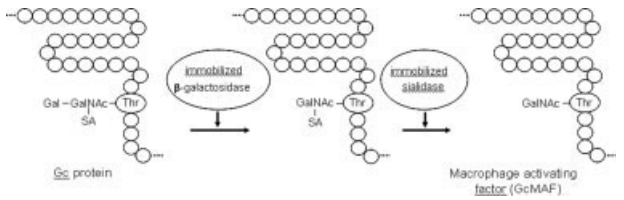

Figure 2 – Stepwise treatment of Gc protein with immobilized β-galactosidase and sialidase to generate GcMAF.

Procedure for preparation of GcMAF

The serum was heat inactivated at 60°C for 1 hr and mixed with 30% saturated ammonium sulfate that precipitates Gc protein fraction.[50] The precipitate was dissolved in PBS (pH 7.4) containing 0.5% Triton X-100 and 0.3% tri-*n*-butyl phosphate, and kept at room temperature overnight to resolve lipid containing microbial contaminants including envelopes viruses if any. The samples were precipitated by 30% saturated ammonium sulfate, dissolved in 50 mM sodium citrate buffer at pH 4.0 and kept overnight. Gc protein was further purified using 25-hydroxyvitamin D_3-affinity chromatography.[49] This procedure isolates Gc protein exclusively. Electrophoretic analysis proved purity of Gc protein (MW 52,000). This chromatographic specificity to Gc protein yields highly pure Gc protein and eliminates all possible contaminations of macromolecules. Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase yielded probably the most potent macrophage activating factor (GcMAF)[23–25,39,41,51] ever discovered (Fig. 2). The immobilized enzymes were removed by centrifugation. The final product, GcMAF, was filtered through low protein-binding filter, Millex-HV, (Millipore Corp., Bedford, MA) for sterilization.

Since the molecule of GcMAF is identical to that of native human MAF, it should have no side effects on humans. In fact numerous administrations (more than 10 times for 3–6 month period) of GcMAF (100–500 ng/human) to 12 humans showed no sign of side effects.[25,28,30,39,40] 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 adhering 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 of breast cancer patients

Participants and eligibility criteria. A group of 16 nonanemic breast cancer patients was included in this study. Patients with mammary adenocarcinoma already treated with the conventional combination therapies; tumor resection (mastectomy or lumpectomy) and radiation or chemotherapy were eligible for GcMAF therapy. Patients received GcMAF therapy exclusively but not combination therapy. Thus, anemic breast 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 The Institutional Review Board, 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,[12,13,39] 100 ng GcMAF was administered intramuscularly once a week.

Procedures to be used for clinical study and study parameters. Serum samples (>2 ml) were periodically (weekly or biweekly) collected immediately prior to each GcMAF administration and used for prognostic analysis. Assessment of patient response to each GcMAF administration was performed by determining both MAF precursor activity of serum Gc protein and serum Nagalase activity during GcMAF therapy. Since serum Nagalase activity is proportional to tumor burden,[25,29,32] kinetic assessment of curative

TABLE I – DIAGNOSTIC AND THERAPEUTIC HISTORY OF BREAST CANCER PATIENTS

Patient		Therapeutic history		Precursor activity[a]	Nagalase[b] (nmole/min/mg)
No.	Age	Surgery	Chemo./Radiat.	Superoxide (nmole)	
1	68	Lumpectomy	Chemotherapy	0.93	3.65
2	63	Lumpectomy	Radiation	2.88	1.96
3	45	Nodectomy Bimastectomy	Radiation Chemotherapy	0.67	4.05
4	70	Mastectomy	Radiation	1.98	2.53
5	46	Mastectomy	Radiation	0.52	4.76
6	46	Mastectomy	Chemotherapy	0.63	4.11
7	58	Mastectomy	Radiation	0.67	4.08
8	77	Mastectomy	Chemotherapy	0.47	6.23
9	44	Lumpectomy	None	2.81	2.24
10	69	Mastectomy	Chemotherapy	2.81	2.24
11	44	Mastectomy	Radiation	1.39	3.19
12	70	Mastectomy	Radiation	1.65	3.05
13	56	Mastectomy	Chemotherapy	0.86	3.69
14	72	Mastectomy	Chemotherapy	2.33	2.44
15	66	Lumpectomy	Chemotherapy	2.01	2.53
16	62	Mastectomy	Chemotherapy	2.04	2.5
C	Healthy human[c]			5.04	0.[d]

[a]Precursor activity <0.85 is unable to support activation of macrophages and that is considered to be loss of activity.–[b]Nagalase assayed before entering GcMAF therapy.–[c]Average of 6 healthy individuals.–[d]This activity level is enzyme activity of α-galactosidase and not of Nagalase.

response to GcMAF therapy was performed by determining serum Nagalase activity as a prognostic index during the entire course of GcMAF therapy for all sixteen patients.

Assay for MAF precursor activity of patient serum Gc protein

Blood samples of healthy humans were collected in tubes containing EDTA to prevent coagulation. The blood samples were generally processed within 2 hr from the time of blood collection. Five milliliter blood sample and 5 ml saline (0.9% NaCl) was mixed and gently laid on a 15-ml centrifuge tube containing 3 ml Lymphoprep™ (similar to Ficoll; Polysciences, Warrington, PA) and centrifuged at 800*g* for 15 min. The dense white cell band of PBMCs containing adhering monocytes (tenacious macrophages) 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 96-mm wells. Incubation for 45 min in a 5% CO_2 incubator at 37°C allowed adherence of macrophages to 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 min. Because of adherence of macrophages to plastic substrata, lymphocytes and macrophages were separately washed with PBS, admixed and cultured in EA medium containing 0.1% serum of breast cancer patients or healthy controls as source of Gc protein. After 3 hr cultivation the extent of macrophage activation was assayed for superoxide generating capacity.[28,29]

The macrophages were washed with PBS and incubated in 1 ml PBS containing 20 μg cytochrome c for 10 min. About 30 min after 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 nmoles 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 are exhibited as a decrease in superoxide generation when compared with control healthy human Gc protein. Thus, this procedure measures the ability of the individual patient to activate macrophages.

However, cultivation of the mixture of lyso-Pc-treated lymphocytes and macrophages in EA medium without containing serum results in production of 0.5–0.8 nmole superoxide/min/10^6 cells.[50,51] Thus, if patient serum (0.1%) generates <0.8 nmole superoxide/min/10^6 cells, the precursor activity of patient serum Gc protein 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 acetate buffer (pH 6.0) and dialyzed against the same buffer at 4°C for 2 hr. The dialysates were made up to 1 ml in volume and assayed for Nagalase activity.[28,29] Substrate solution (250 μl) contained 1.5 μmoles of *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide in 50 mM acetate buffer (pH 6.0). The reaction was initiated by addition of 250 μl of the dialyzed samples, kept at 37°C for 60 min and terminated by adding 200 μl of 10% TCA. After centrifugation of the reaction mixture, 300 μl of 0.5 M Na_2CO_3 solution was added to the supernatant.[28,29] The amount of released *p*-nitrophenol was determined spectrophotometrically at 420 nm and expressed as nmole per minute per mg protein.[28,29] Protein concentrations were estimated by the Bradford method.[52]

The half-life of Nagalase activity *in vivo* is less than 24 hr as we observed sudden drop of Nagalase activity 24 hr post-resection of the tumor.[29,30] However, Nagalase activity in serum is extremely stable and highly reproducible after storage of sera at 4°C for more than several months.[29,30] Serum Nagalase activity is proportional to tumor burden as determined by tumor weight and cell counts.[29,32]

Healthy control sera exhibit low levels (0.35–0.69 nmoles/min/mg) 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.[28,29,33] When serum enzyme activity of patients during GcMAF therapy reaches to approximately 0.69 nmoles/min/mg or less, serum Nagalase (as for tumor burden) is eradicated.

Results

Therapeutic history and diagnostic parameters of metastatic breast cancer patients

Table I summarizes the therapeutic history of 16 breast cancer patients. All patients received surgical resection by either mastectomy or lumpectomy. All except 1 of these patients have been treated with radiation or chemotherapy after tumor resection. Because the fate and staging of the malignant disease correlate with the degree of immunosuppression and tumor burden,[28,29] before entering GcMAF therapy the MAF precursor activity of Gc protein and serum Nagalase activity for each patient were determined regardless the lapse of time after tumor resection and adjuvant therapy.

TABLE II – CORRELATION BETWEEN THE MAF PRECURSOR ACTIVITY AND SERUM NAGALASE ACTIVITY OF 5 BREAST CANCER PATIENTS DURING GcMAF THERAPY

Patient no	Time assayed week	Precursor activity superoxide (nmole)	Nagalase (nmole/min/mg)
1 (10)[a]	0	2.81	2.24
	1	2.89	1.98
	4	3.04	1.85
	8	3.63	1.06
	14	4.01	0.93
	17	4.21	0.73
	22	4.44	0.69
2 (13)	0	0.86	3.69
	1	1.93	2.85
	4	1.95	2.66
	6	2.21	2.43
	9	2.43	2.27
	12	2.97	1.99
	15	3.21	1.67
	19	3.43	1.54
	22	4.05	0.62
3 (11)	0	1.39	3.19
	1	1.65	3.01
	3	2.00	2.92
	4	2.23	2.67
	5	2.51	2.37
	6	2.61	1.97
	11	3.14	1.39
	18	3.82	1.46
	22	4.41	0.68
4 (2)	0	2.88	1.96
	2	3.04	1.47
	4	3.21	1.29
	6	3.36	0.99
	9	3.61	0.89
	12	4.11	0.72
	20	4.78	0.63
5 (16)	0	2.04	2.95
	1	2.46	2.50
	2	2.57	2.26
	4	2.83	1.88
	6	3.21	1.42
	9	3.39	1.21
	12	3.5	
	15	3.64	0.94
	18	4.1	0.82
	21	4.	0.67

[a]The number refers to the patient number of Table I.

Since macrophage activation for phagocytosis and subsequent antigen presentation to B and T cells is the first indispensable step for humoral and cellular immunity development,[28,29,35,39,40] lack of macrophage activation leads to immunosuppression. Thus, the MAF precursor activity of patient serum Gc protein was first to be examined. As shown in Table I, the MAF precursor activities of patient Gc protein were lost or reduced. Because loss or reduction of the MAF precursor activity of patient Gc protein results from deglycosylation of the Gc protein by serum Nagalase secreted from cancerous cells,[28,29] patient serum Nagalase activities were examined. Patients having lower precursor activity of the Gc protein showed a higher serum Nagalase activity (Table I). Since serum Nagalase activity is directly proportional to tumor burden,[28–31,39] the serum Nagalase activity estimates the total amount of metastasized tumor cells in these tumor-resected patients. Thus, serum Nagalase activities of individual patients were presented before entering GcMAF therapy and used as a baseline control for prognostic analysis of serum Nagalase during GcMAF treatment.

Prognostic parameters for breast cancer during GcMAF therapy

The reduced MAF precursor activity of individual cancer patients provides an indication of their reduced immune potential that is the major factor for cancer patient survival.[29] Time course study of the MAF precursor activity of 5 breast cancer patients (nos. 2, 10, 11, 13 and 16) was conducted during GcMAF therapy.

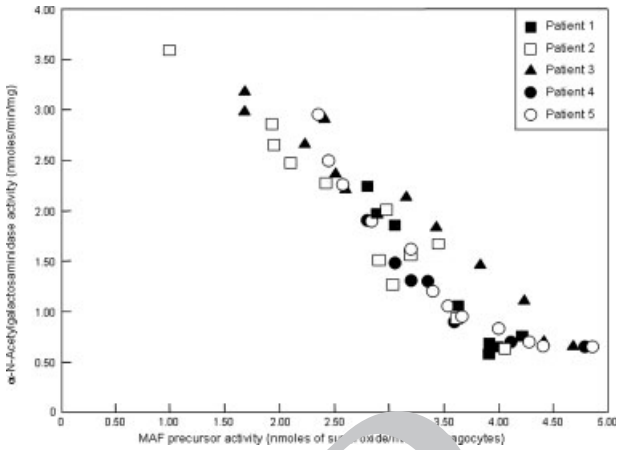

FIGURE 3 – Inverse correlation between the MAF precursor activity of serum Gc protein and serum α-N-Acetylgalactosaminidase (Nagalase) activity of breast cancer patients during GcMAF therapy.

The initial MAF precursor activities of all 5 patients were ranging from 0.86 to 2.86 nmoles of superoxide produced/min/10^6 macrophages. The MAF precursor activity of all 5 patients quickly increased as GcMAF therapy progressed as shown in Table II. After less than 22 administrations of GcMAF, their MAF precursor activity increased up to > 5 nmoles of superoxide produced/min/10^6 macrophages. The result suggests that these patients improved their immune potential rapidly during GcMAF therapy. Therefore, patient tumor burden ought to be estimated during the therapy. Because of proportionality of serum Nagalase activity to tumor burden, the serum Nagalase activity of these patients were analyzed during GcMAF therapy. As the MAF precursor activity increased, serum Nagalase activities of all 5 patients decreased toward the healthy control level (Table II). When the MAF precursor activities of individual patients were compared with their serum Nagalase activities during GcMAF therapy, an inverse correlation between these parameters of all 5 patients was evident as shown in Table II.

To illustrate quantitative correlation of these prognostic parameters, the time course precursor activities of individual breast cancer patients were plotted against their serum Nagalase activities. Figure 3 shows that as GcMAF therapy progress the MAF precursor activity increased with a concomitant decrease in serum Nagalase activity. These prognostic parameters of all 5 individual patients fell in the same linear inverse correlationship. As the MAF precursor activity increased toward the healthy control value, serum Nagalase activities of these patients decreased toward the healthy control level (Table II and Fig. 3). Since serum Nagalase activity of metastatic breast cancer patients is directly proportional to the total amount of metastasized tumor cells,[28–31,39] the amount of tumor cells decreased rapidly as GcMAF therapy progressed.

Time course study of serum nagalase activity of 16 breast cancer patients during GcMAF therapy

Because of precision of serum Nagalase assay,[29–31] the entire time course of GcMAF therapy of breast cancer patients should be monitored by serum Nagalase activity as a prognostic index. These patients had the initial Nagalase activities ranging from 1.96 to 6.23 nmole/min/mg. As shown in Figure 4, the serum Nagalase activities of all sixteen patients decreased as GcMAF therapy progressed. After 18–22 administrations (18–22 weeks) of 100 ng GcMAF, all 16 patients had very low serum Nagalase activity levels equivalent to those of healthy control values less than 0.69 nmole/min/mg. The result indicates that these patients are free of cancer cells.

During 4 years after completion of GcMAF therapy, biannual serum Nagalase assays of all 16 breast cancer patients showed no increase over the healthy control serum enzyme activity levels ranging from 0.45 to 0.69 nmole/min/mg. Biannual assays of CEA

values of these patients also showed the normal healthy values ranging from 1.1 to 1.7 ng/ml for the entire 4-year period. Furthermore, annual CAT scans of these patients confirmed them being tumor-free.

Curative rate of breast cancer during GcMAF therapy depends on cell membrane abnormality

The highly activated macrophages developed enormous variation of receptors[38] that recognize their abnormalities on the malignant cell surface and rapidly kill cancerous cells with more membrane abnormalities in poorly differentiated (termed undifferentiated) cancer cells than less membrane abnormality in moderately or immediately differentiated (differentiated for short) tumor cells. For example, patient nos. 1–5, 8–13, 15 and 16 in Figure 4, their serum Nagalase activities rapidly decreased during the first few weeks (up to 4 weeks) and followed by slow decrease for the remaining 12–18 weeks. The result suggests that their cancer cell populations are a mixture of cells with 2 different levels of membrane abnormality: undifferentiated cells and differentiated cells. These mixed populations of undifferentiated and differentiated tumor cells are likely to be developed by differentiation from the undifferentiated cells during tumor growth. In contrast the serum Nagalase activities of patient nos. 6, 7 and 14 slowly decreased during the entire 22 weeks therapeutic period. The result suggests that their cancer cell populations are already differentiated without further differentiation, because the rate of the decrease in serum Nagalase in these no. 6, 7 and 14 patients is much slower than that of the undifferentiated tumor populations of the aforementioned tumors. The mixed tumor population of differentiation seems to be common in a variety of cancers. A similar kind of pattern of serum Nagalase activity during GcMAF therapy of the mixed differentiation levels of tumors is found in GcMAF therapy of prostate cancer patients.[40]

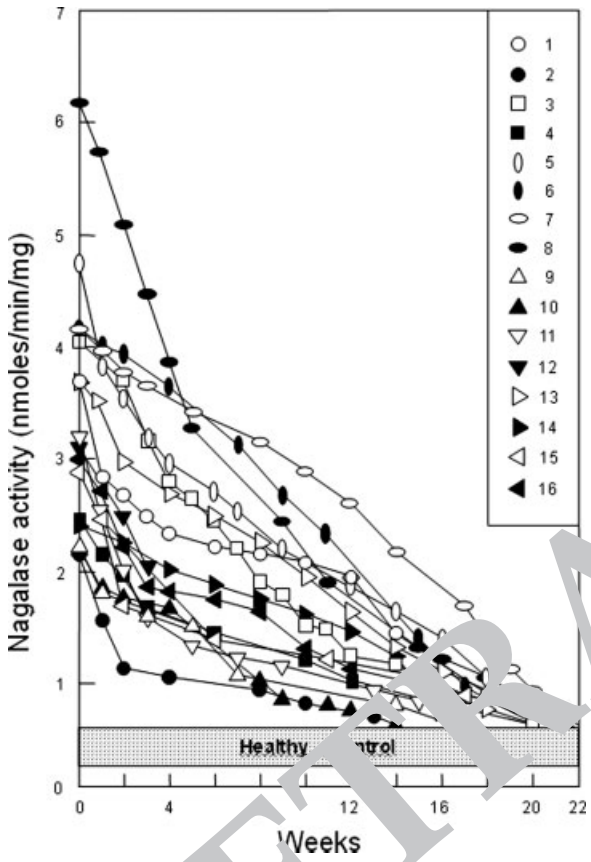

FIGURE 4 – Time course of GcMAF therapy of 16 metastatic breast cancer patients with serum Nagalase activity as a prognostic index.

Correlation between serum Nagalase activity and breast tumor marker levels during GcMAF therapy

Since serum Nagalase activity is an excellent index for estimation of tumor burden, activity levels of 3 breast tumor markers, CA27.29, CA15-3 and CEA, were compared with serum Nagalase activity during GcMAF therapy of 4 patients with mastectomy as shown in Table III. As serum Nagalase decreased during GcMAF therapy, all other breast tumor markers decreased.

Discussion

Administration of GcMAF to cancer patients is a very effective procedure for cancer therapy in spite of the presence of high Nagalase activity in patient sera. Although Gc protein is efficiently deglycosylated by serum Nagalase secreted from cancerous cells[28,29,32,37] (Fig. 1*b*), serum Nagalase has no effect on GcMAF. Thus, the capacity of the enzyme to remove the trisaccharide of the glycoprotein indicates that serum Nagalase is endo-Nagalase.[31] When GcMAF was added to cancer patient serum containing a high Nagalase activity and incubated for 4 hr at 37°C, the potency of GcMAF activity did not decrease.[29] These results indicate that Nagalase under colloidal serum environment, known as oncotic pressure,[53] acts as endo-Nagalase but not as an exo-enzyme because Nagalase in serum is unable to deglycosylate a

TABLE III – CORRELATION BETWEEN SERUM NAGALASE ACTIVITY AND BREAST CANCER TUMOR MARKERS CA27.29, CA15-3 AND CEA OF 4 PATIENTS DURING GCMAF THERAPY

Patient No.	GcMAF therapy week	Nagalase (nmole/min/mg)	CA27.29 (<37.5)[a]	CA15-3 (<30)	CEA (<30)
M1	0	2.24	295.5	181.0	11.8
	4	1.80	120.8	69.8	9.6
	14	0.93	35.0	28.5	2.5
	20	0.75	26.4	27.3	2.7
	23	0.69	25.0	26.9	1.2
M2	0	3.85	377.9		23.4
	4	2.22	173.5		10.4
	10	1.26	62.7		4.0
	22	0.74	28.2		1.4
M3	0	2.15		143.5	9.8
	6	1.19		57.6	8.5
	12	0.84		28.2	2.8
	21	0.65		23.6	1.2
M4	0	1.98		105.0	9.7
	6	1.29		51.3	2.4
	14	0.74		24.3	2.3
	20	0.69		20.6	1.5

Units for CA 27.29 and CA 15-3 are U/ml and unit for CEA is ng/ml.
[a]Parenthesized numbers indicate the range of healthy control values.

monosaccharide, *N*-acetylgalactosamine (GalNAc), of GcMAF. Administration of GcMAF (100 ng) to healthy humans and advanced cancer patients results in the same extent of macrophage activation, confirming that serum Nagalase has no effect on the potency of GcMAF. However, under nononcotic salt buffered medium, Nagalase activity can be measured as an exo-enzyme activity with a readily available chromogenic substrate, *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide. This Nagalase assay procedure allows us to use it for prognosis of all types of malignant diseases.

Because the half-life of the activated macrophages is about 6 days,[13,14,39] 100 ng GcMAF was administered intramuscularly once a week.[30,39] Shortly after administration of GcMAF to cancer patients GcMAF spreads systemically including tumor tissues. However, GcMAF cannot activate the tumor associated macrophages (TAM as type II), because TAM had lost the receptor for MAF (unpublished data). In contrast, systemically activated macrophages exhibited greatly enhanced phagocytic and superoxide generating capacities in 3.5 hr after GcMAF administration to patients, regardless of the stage of cancer.[25] Although cancer patients carry immature myeloid cells,[54] GcMAF can readily convert them to activated macrophages (unpublished data). 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.[25,51] Such highly activated systemic macrophages are chemotactically recruited to inflamed lesions by 180-fold increase of the macrophage cell counts.[51]

Macrophages, activated by GcMAF, were found to immediately (less than 30 min) bind cultured cells of 1 prostate, 3 breast, 2 colon, 2 oral and 1 ovarian cancer cell lines. The GcMAF activated macrophages rapidly phagocytize various bacteria (*E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *B.subtilis*, *Clostridium perfringens* and *Mycobacterium tuberculosis*) and various viruses (rubella, measles, herpes simplex, parainfluenza (Sendai), influenza virus and HIV type 1) (unpublished data). Macrophage scavenger receptors recognize a number of ligands, including chemically modified or altered molecules, in particular, modified lipoproteins as well as a number of microorganisms.[55] Taylor *et al*.[56] reviewed that macrophages express a broad range of plasma membrane receptors that mediate the interaction with natural and altered-self components (*e.g*., abnormalities) of the host as well as a range of microorganisms. Thus, the macrophages activated by GcMAF developed normal variation of receptors[38] that recognize a variety of microbial agents (*e.g*., bacteria and viruses) and abnormalities in malignant cell surfaces. This fundamental nature of macrophages to recognize cell surface abnormality (nonselfing nature) is universal to all types of cancers. In fact weekly administration of [illegible]ng GcMAF to cancer patients showed curative effects on a variety of cancers.[30,32,37–40] Types of cancer so far tested are prostate, breast, colon, stomach, liver, lung (including mesothelioma), kidney, bladder, uterus, ovarian, head/neck, brain cancer, melanoma and fibrosarcoma.[30,38–40] Efficacy of GcMAF therapy for cancers depends on the degree of cell membrane abnormality. 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. In fact adenocarcinoma 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. This curative rate appears to depend on both the amount of receptors for the particular antigen on macrophages and the amount of antigens on each cell. However, many tumors contain the mixed population of undifferentiated and differentiated cells. In fact during GcMAF therapy of the majority of breast cancer patients, serum Nagalase activity rapidly decreased during the first few weeks and slowly decreased for the rest of the therapeutic period. Therefore, the significance of GcMAF therapy of breast cancer has been greatly enhanced by the discovery of cancer cell-specific Nagalase that can be accurately monitored for the rate of regression during GcMAF therapy.[28–32]

GcMAF does not activate dendritic cells (unpublished data). Thus, therapeutic administration of GcMAF to cancer patients activates macrophages exclusively. The activated macrophages are rapidly engaged in tumoricidal action and processing the ingested antigens that in turn empowers them to become highly active antigen-presenting cells and key players in development of adaptive immunity as in the present study. Of the 2 major pathways of adaptive immunity development, the GcMAF activated macrophages present the processed antigens *via* HLA-II antigen complex-mediation to CD4+ T cells appears to preferentially engage B cells. Consequently, large amounts of antibodies are produced and develop preferentially tumor immunity against the cancer. In fact, simultaneous administration of 50 pg GcMAF and sheep red blood cells to mice produces a large amount of antibody secreting cells in 2 days (48 hr) post administration of GcMAF as demonstrated by Jerne plaque assays.[51] Thus, GcMAF has a potent immuno-adjuvant activity. Six days after immunization of GcMAF-treated mice with heat-killed Ehrlich ascites tumor cells, the same ascites tumor cells no longer transplantable in these mice.[51] In the present human breast cancer model, GcMAF (100 pg/ml)-treated macrophages are highly tumoricidal to breast cancer cells.[38,40] After GcMAF therapy of breast cancer patients, antibodies (IgG) against the abnormality (breast cancer antigen) are produced.[38,40,57] This was demonstrated by the rapid tumor cell killing (trypan blue exclusion test) by *in vitro* GcMAF-treated macrophages in the presence of GcMAF-treated patient serum (or IgG fraction). Approximately 85% of human mammary carcinoma cells (MDA-MB-231 and MCF7) were killed by GcMAF-treated macrophages in the presence of GcMAF-treated breast cancer patient serum (or IgG fraction) in 4 hr but in the absence of patient serum in 18 hr.[40] These results suggest that the activated macrophages kill the cancer cells preferentially *via* Fc-receptor mediation.[38,40] This is because GcMAF-treated macrophages develop a large amount of Fc-receptors.[8,9,25]

There are 2 major immunotherapeutic procedures for cancer; vaccine therapy and monoclonal antibody therapy which have been investigated for nearly 30 years. Since macrophage activation and subsequent antigen processing are indispensable steps for development of immunity against vaccines, cancer vaccine therapy requires activation of macrophages for development of T or B cell mediated tumor rejection. Monoclonal antibody therapy also requires activation of macrophages for development of tumoricidal activity *via* Fc-receptor mediation and further humoral immunity. Thus, these immunotherapies require activation of macrophages to be effective. The importance of focusing cancer immunotherapy on macrophage activation has been clearly demonstrated in the present study.

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